

INTACT



Project no.: LSHC-CT-2003-506803

Project acronym: INTACT

Project Title: Identification of Novel Targets for Cancer Therapy

Instrument: Integrated Project

Thematic Priority: Life sciences, genomics and biotechnology for health

3rd Annual Periodic Activity Report

Period covered: 1.1.2006-31.12.2006

Date of preparation:
15.2.2007

Start date of project: 1.1.2004

Duration: 4 years

Project coordinator organization name: University of Copenhagen (UKBH)

Project coordinator name: Kristian Helin

INTACT research coordinator (editor) name: Pia Klausen

INTACT financial manager name: Johanna Tara Bowns Poulsen

Publishable executive summary	4
Section 1 – Project objectives and major achievements during the reporting period	7
Section 2 – Workpackage progress in the period (2006)	9
SUMMARY of research activities during 2006 Cluster 1 (WPs 1-6): Technology Platforms (NKI2 (coordinator), NKI3, ISREC2)	9
Cluster 1: WP 1 Technology development for bar coded RNAi screens (NKI2)	10
Cluster 1: WP 2 Generation of the murine RNAi vector library (NKI2)	11
Cluster 1: WP 3 Development of ERMtag screens to identify pathway-specific cancer genes in mammalian cells (NKI3)	12
Cluster 1: WP 4 Development of Retrovirus-based living cell microarrays (IEO2)	13
Cluster 1: WP 5 Activity D. Lentiviral based pathway regulation in <i>in vivo</i> mouse models (NKI1)	17
Cluster 1: WP 6 Establishment of a mouse xenograft platform to validate putative cancer therapy targets (ISREC2)	21
SUMMARY of research activities during 2006 Cluster 2 (WPs 7-13): DNA damage and p53 (UKBH, CNIO1 (coordinator), BICR, NKI2, IEO2)	27
Cluster 2: WP 7 Targeting the ATM-ATR/CHK1-CHK2 pathway using RNAi and ERMtag-based screens (UKBH)	28
Cluster 2: WP 8 Telomere damage response (CNIO1)	31
Cluster 2: WP 9 Role of telomerase in tumour promotion and metastasis (CNIO1)	33
Cluster 2: WP10 Targeting the p53 pathway using RNAi and ERMtag-based screens (BICR (BICR/CNIO2))	35
Cluster 2: WP 11 Identification of novel regulators of PUMA expression (BICR)	39
Cluster 2: WP 12 Identification of synthetic lethal interactions with p53 (NKI2)	42
Cluster 2: WP 13 Identification of activators of the p53 pathway in cells expressing haematopoietic oncogenic kinases (IEO2)	45
SUMMARY of research activities during 2006 Cluster 3 (WPs 14-17): The pRB Pathway (NKI3, UKBH, CNIO1, IEO2 (coordinator))	48
Cluster 3: WP14 Development of ERMtag screens to identify pathway-specific cancer genes in mammalian cells (NKI3)	50
Cluster 3: WP 15 Targeting E2F using RNAi and ERMtag-based screens (UKBH)	53
Cluster 3: WP 16 Identification of epigenetic regulators of telomere function CNIO1	55
Cluster 3: WP 17 Synthetic lethality with Rb loss and p53-independent checkpoints IEO	58
Generation of human normal fibroblasts (WI38) with impaired Rb function through stable expression of a retroviral construct that express RNAi specific for Rb.	60
SUMMARY of research activities during 2006 Cluster 4 (WPs 18-20): Ras/Raf Pathway and cellular senescence (GEM, Pasteur, CNIO2 (coordinator))	61
Cluster 4: WP 18 Targets of the Raf pathway in tumourigenesis in vivo and in vitro. (GEM)	62
Cluster 4: WP 19 Deciphering PML- and PIASy- Induced Senescence (Pasteur)	67
Cluster 4: WP 20 Identification of novel tumour suppressor pathways (CNIO2)	70
SUMMARY of research activities during 2006 Cluster 5 (WPs 21-23): The MYC Pathway (UNIMAR, ISREC1 (coordinator))	73
Cluster 5: WP 21 Targeting the Myc/Miz1 pathway using RNAi and ERMtag-based screens (UNIMAR)	74
Cluster 5: WP 22 Identification of genes that show synthetic lethality with deregulation of the Myc/Miz pathway (UNIMAR)	80
Cluster 5: WP 23 Identification of genes that promote long-term self-renewal of hematopoietic stem cells carrying oncogenic mutations in the ARF, Myc or PI3-kinase pathways (ISREC1)	84
SUMMARY of research activities during 2006 Cluster 6 (WPs 24-26): Identification of new targets by synthetic lethality screens of preexisting tumor models (NKI2, MDC (coordinator), NKI1)	88
Cluster 6: WP 24 Bar coded RNAi screens to identify synthetic lethal interactions in mammalian cells (NKI2)	90
Cluster 6: WP 25 Screening for drug resistance in primary lymphomas in vitro and in vivo (MDC)	92
Cluster 6: WP 26 The role of distinct cancer pathways in the resistance to therapy (NKI1)	96
Additional workpackages (WPs 27-32)	99

Third Annual Periodic Activity Report (2006)

WP 27 Systematic analysis of gene expression in primary human tumors. (Agendia)	99
WP 28 Morphochem – workpackage ended	101
WP29 Management activities (UKBH)	102
WP 30 CGTM activities (GEM)	103
WP 31 Management activity, Web presence (UNIMAR)	104
WP 32 Study of synthetic lethality on Myc overexpressing cell lines (Vichem)	105
Section 3 – Consortium management	113
List of deliverables (months 1-42)	113
List of milestones (months 1 – 42)	127
Table 5 Workpackages – Plan and Status Barchart	139
Section 4 – Other issues	152
Section 5 – Plan for using and disseminating of knowledge (PUDK)	153
Partner 1, UKBH (WP 7, 15)	153
Partner 2, NKI1 (WP 5, 26)	158
Partner 2, NKI2 (WP 1, 2, 12, 25)	160
Partner 2, NKI3 (WP3, 14)	163
Partner 3, CNIO1 (WP 8, 9, 16)	167
Partner 3, CNIO2 (WP 20)	174
Partner 4, GEM (WP 18)	180
Partner 5, MDC (WP 25)	182
Partner 6, UNIMAR (WP 21, 22)	184
Partner 7, ISREC1 (WP23)	187
Partner 7, ISREC2 (WP 6)	190
Partner 8, BICR (WP 10, 11)	191
Partner 9, Pasteur (WP 19)	193
Partner 10, MCH (WP 28) –closed	195
Partner 11, Agendia (WP 27)	196
Partner 12, IEO (WP 4, 13, 17)	197
Partner 13, Vichem (WP 32)	203

Publishable executive summary

In INTACT we have proposed to develop and apply new functional genomics technologies that will provide unique approaches to the design of new pathway-specific cancer therapies.

Our specific objectives are:

- To develop large-scale functional genomic analysis to identify novel mechanisms involved in cancer development; specifically, we will generate the tools and technologies to carry out genome-wide loss-of-function screens in mammalian cells.
- To apply these technologies to specific models of major human cancer-causing pathways to define novel targets for therapy.
- To use existing and generate novel mouse models and non-invasive tumour imaging to validate and assay cancer gene function *in vivo* and to develop new treatment modalities.
- To develop cell-based assays for cancer-relevant genes and pathways that will serve as readout for the identification of anticancer agents through the screening of chemical compound libraries.
- To distribute and disseminate the novel technologies for the study of gene function *in vitro* and *in vivo* within the consortium and to researchers in the European Community.

To reach these objectives we have formed a multidisciplinary research consortium, including top scientists with extensive experience in developing innovative genomics technologies and with an excellent track-record in identifying key signalling molecules involved in cancer, as well as SMEs with experience in identifying cancer-relevant genes and in screening chemical compound libraries. The consortium has 16 participants from 12 institutions/companies. The contractors are:

- BRIC, University of Copenhagen, Copenhagen, Denmark (partner 1: Kristian Helin (UKBH))
- The Netherlands Cancer Institute, Amsterdam, The Netherlands (partner 2: Anton Berns (NKI1), René Bernards (NKI2) and Maarten van Lohuizen (NKI3))
- The Spanish National Cancer Center, Madrid, Spain (partner 3: Maria Blasco (CNIO1) and Manuel Serrano (CNIO2))
- University of Vienna, Vienna, Austria (partner 4: Manuela Baccarini (GEM))
- Max Delbrück Center for Molecular Medicine; Berlin, Germany (partner 5: Clemens Schmitt (MDC))
- University of Marburg, Marburg, Germany (partner 6: Martin Eilers (UNIMAR))
- Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland (partner 7: Andreas Trumpf (ISREC1) and Michel Auguet (ISREC2))
- Beatson Institute for Cancer Research, Glasgow, United Kingdom (partner 8: Karen Vousden (BICR))
- Pasteur Institute, Paris, France (partner 9: Anne Dejean (Pasteur))
- Agendia N.V., Amsterdam, The Netherlands (partner 11, SME [Agendia])
- European Institute of Oncology, Milan, Italy (partner 12: Pier Giuseppe Pelicci (IEO2))
- Vichem Chemie Ltd., Budapest, Hungary (partner 13, SME [Vichem]).

The coordinator of INTACT is Professor Kristian Helin, BRIC, University of Copenhagen. Contact details are: Prof. Kristian Helin, Biotech Research & Innovation Centre (BRIC), Ole Maaløes Vej 5, DK-2200 Copenhagen, Denmark. Phone: +45 3532 5666; Fax: +45 3532 5669; e-mail: kristian.helin@bric.dk. INTACT research coordinator and editor of reports is Pia Klausen, Ph.D. at BRIC, phone: +45 3532 5648 e-mail: pia.klausen@bric.dk.

Third Annual Periodic Activity Report (2006)

In INTACT we have defined a technology and a scientific platform. The main objectives of the technology platform is to develop technologies to screen shRNA libraries (among them bar-code screens and living cell microarrays), to generate a mouse shRNA library, to develop highly efficient insertional mutagenesis screens, to develop a system to efficiently generate mice with knockdowned expression of cellular genes using lentivirus technology, and to generate a xenograft platform. The main objective of the scientific platform is to use the technologies to identify and characterize novel genes with relevance for cancer therapy. To do this, a number of cell-based assays will be (and have been) designed to identify cancer relevant genes by screening of shRNA libraries and by the use of insertional mutagenesis screens. Furthermore existing and new mouse models will be used to identify and characterize novel cancer relevant genes, and to assess and develop novel treatments for cancer.

In the first 36 months of INTACT we have defined the following main milestones for the technology platform:

1. Establishment of bar-code screen for human shRNA library
2. Generation of 30,000 clones for the mouse shRNA library
3. Generation of vectors for ERM-tag screens
4. Generation of novel lentivirus vectors for the conditional expression of shRNA constructs and protocols for making transgenic mice
5. Establishment of cellular models for performing ERM-tag and shRNA library screens
6. Establishment of “living cell microarray”
7. Establishment of High Content Screening systems

One of the central components of the technology cluster is the generation of shRNA libraries and technology to facilitate the screening of these libraries (bar code technology and living cell microarrays). The development of the bar code technology has been completed successfully, protocols have been disseminated to the INTACT participants and 2 workshops have been organized for the participants of INTACT and one also for participants of TRANSFOG (another EU-FP6 integrated project). The existing human shRNA library has been distributed to the consortium partners and they have obtained “hits” using the library (see also below). Furthermore, the mouse shRNA library has been completed. The Sanger Centre in Hinxton (UK) has sequenced the human shRNA library, and approximately 12,500 sequence-verified shRNA constructs will be distributed to the partners of INTACT in the spring of 2007. This library will also be made available for the scientific community.

The establishment of High Content Screening (HCS) systems was not part of the original technology platform. However, several of the partners of INTACT realised that the ability to set up imaging or selection screens in 96- and 384-well format would be an advantage in several screenings and would be superior to the “living cell microarray”, because it is easier and more flexible to work with this technology. This has been a major commitment of several of the partners, however, we believe that it is the best current available technology and expect that several novel candidate genes involved in the development of cancer will be identified using HCS.

The following main milestones and achievements have been achieved for the scientific platform in the first 36 months.

1. Identification of novel regulators of telomere length, the G2 DNA damage checkpoint, regulators of p53 activity, genes showing synthetic lethality with p53, regulators of PML-induced senescence, regulators of the Myc/Miz1 pathway, and genes that confer resistance to SAHA and Nutlin (2 experimental cancer agents).

Third Annual Periodic Activity Report (2006)

2. Implementation of in vivo systems in addition to the ones used above to identify regulators of telomerase activity, heterochromatin structure, p53 activity, the Sonic Hedgehog pathway, the pRB-E2F pathway, oncogene-induced senescence and drug-induced senescence.
3. Identification of a 54-gene signature that identifies a high-risk subgroup in non-small lung carcinoma patients. In addition, completion of gene expression profiling for melanoma and older breast cancer patients.
4. Implementation of screens for compounds that show synthetic lethality with lack of p53.

The partners are now characterizing the identified genes. This characterization also includes validation of the genes as potential targets for the development of novel anti-cancer drugs.

The INTACT web page is active and updated with information regarding open positions and events. Furthermore, it contains a password-protected collection of protocols for INTACT members only.

In summary INTACT is on track and we foresee that the next year will be very exciting. We will see several publications based on projects started within the INTACT programme. Moreover, more genes will be identified with a potential role in the development of human cancer. Mouse models will be generated and compounds with anti-cancer activity will be identified.

We therefore expect that INTACT will reach its long-term goals, i.e. to distribute novel technologies for the study of gene function in vitro and vivo to researchers in the European Community, to identify novel genes with relevance for cancer, to suggest novel treatment modalities for cancer, and in collaboration with Biotech and pharmaceutical industry set-up screenings for the identification of novel drugs for the treatment of cancer patients.

Section 1 – Project objectives and major achievements during the reporting period

The major project objectives of INTACT are:

To develop large-scale functional genomic analysis to identify novel mechanisms involved in cancer development; specifically, to generate the tools and technologies to carry out genome-wide loss-of-function screens in mammalian cells.

To apply these technologies to specific models of major human cancer-causing pathways to define novel targets for therapy.

To use existing and generate novel mouse models and non-invasive tumour imaging to validate and assay cancer gene function *in vivo* and to develop new treatment modalities.

To develop cell-based assays for cancer-relevant genes and pathways that will serve as readout for the identification of anticancer agents through the screening of chemical compound libraries.

To distribute and disseminate the novel technologies for the study of gene function *in vitro* and *in vivo* within the consortium and to researchers in the European Community.

To achieve these objectives we have defined a technology and a scientific platform. The main objectives of the technology platform is to develop technologies to screen shRNA libraries (among them bar-code screens and living cell microarrays), to generate a mouse shRNA library, to develop highly efficient insertional mutagenesis screens, to develop a system to efficiently generate mice with knockdowned expression of cellular genes, and to generate a xenograft platform. The main objective of the scientific platform is to use the technologies to identify and characterize novel genes with relevance for cancer therapy. To do this, a number of cell-based assays have been designed to identify cancer relevant genes through the screening of shRNA libraries. Furthermore existing and new mouse models will be used to identify and characterize novel cancer relevant genes, at to assess and develop novel treatments for cancer.

In the first 36 months we have reached the following main milestones:

Establishment of bar-code screen for human shRNA library

Generation of 30,000 clones for the mouse shRNA library

Generation of vectors for ERM-tag screens

Generation of novel lentivirus vectors for the conditional expression of shRNA constructs and protocols for making transgenic mice

Establishment of cellular models for performing ERM-tag and shRNA library screens

Establishment of “living cell microarray”

The human shRNA library was constructed prior to the start of INTACT. However, since we discovered that propagation of the plasmids frequently led to the deletion of the inserts, we generated a novel shRNA expression vector and recloned the whole library. Furthermore we collaborated with the Sanger Centre, which agreed to sequence the human shRNA library, and we are very pleased that we now have a set of approximately 12,500 shRNA constructs. This library will be made available to the scientific community.

For the scientific platform the following main milestones and achievements have been reached in the first 36 months.

Identification of novel regulators of telomere length, the G2 DNA damage checkpoint, regulators of p53 activity, genes showing synthetic lethality with p53, regulators of PML-induced senescence, regulators of the Myc/Miz1 pathway, and genes that confer resistance to SAHA and Nutlin (2 experimental cancer agents).

Third Annual Periodic Activity Report (2006)

Implementation of in vivo systems in addition to the ones used above to identify regulators of telomerase activity, heterochromatin structure, p53 activity, the Sonic Hedgehog pathway, the pRB-E2F pathway, oncogene-induced senescence and drug-induced senescence.

Identification of a 54-gene signature that identifies a high-risk subgroup in non-small lung carcinoma patients. In addition, completion of gene expression profiling for melanoma and older breast cancer patients.

Implementation of screens for compounds that show synthetic lethality with lack of p53.

The partners are now characterizing the identified genes. This characterization also includes validation of the genes as potential targets for the development of novel anti-cancer drugs.

Some of the scientific work in the workpackages (WPs) has been delayed compared to the initial plans. These delays are by and large a result of technical or biological obstacles, which is quite common in the type of science and technology development done in INTACT. The scientists in charge are working on alternative approaches to tackle the scientific and technological issues, and the participants in INTACT are therefore making significant progress in all the proposed WPs.

In summary INTACT is on track. The major milestones have been achieved or are only slightly delayed, and we foresee that the next year will be very exciting. We will see a number of publications as a result of science started in INTACT, experiments to validate some of the newly identified targets as drug candidates and the identification of the first novel anti-cancer molecules.

Section 2 – Workpackage progress in the period (2006)

SUMMARY of research activities during 2006 Cluster 1 (WPs 1-6): Technology Platforms (NKI2 (coordinator), NKI3, ISREC2)

The major task of cluster 1 of the INTACT program is the development of new technology to study cancer-relevant pathways. A central component of the technology cluster was the generation of shRNA libraries and technology to facilitate the screening of these libraries by partner NKI2 (bar code technology). The libraries have been generated, development of the bar code technology has been completed successfully, protocols have been disseminated to the INTACT participants and two teaching courses for INTACT members in bar code technology have been organized in Amsterdam. The existing human shRNA library and newly generated mouse shRNA library (NKI2) have been distributed to the consortium partners and several “hits” in genetic screens described in the other work packages have been identified by consortium partners. Two joint manuscripts resulting from these INTACT collaborations have been submitted for publication in 2006 and should be published in 2007.

The ERM-TAG project (WP3) has been successfully completed and has yielded a proof-of-concept positive result, showing the feasibility of the approach (WP3, NKI3).

WP4 (IEO) successfully spotted lentiviral vectors on a microarray, and subsequently managed to infect a variety of cells that were seeded on the array, demonstrating feasibility of the “living cell microarray” concept with lentiviral vectors. However, the high throughput production of retroviral supernatants has proven to be challenging. The need to develop such “living cell microarray” has been reduced during the course of the INTACT program, as the development of high throughput reverse transfection of siRNAs has been shown to be efficient in transfecting a variety of cell types. IEO has therefore changed the focus of the project to use the spotting technology to develop a “immunocell array” with spotted antibodies against a large number of proteins. Such arrays allow the detection and subcellular localization of a large number of proteins in parallel. Proof of concept has been reached.

WP5 (NKI1) made good progress towards the stated goals. A doxycyclin-regulatable shRNA vector was developed using new technology (miRNA in combination with a polymerase type II promoter), which works very well in cell culture. The testing in transgenic animals is planned for the last year. A reporter construct that measures activation state of the p53 pathway was also successfully completed with proof of concept in cell lines. Again, the transfer of the vectors to animal models is ongoing and should be completed in the last year.

WP6 (ISREC2) managed to knock down beta-catenin expression in colorectal cancer cell lines in vitro and in vivo. However, knockdown in vivo was heterogeneous, with the cells having efficient knockdown showing a more differentiated phenotype. This issue is still not resolved. Orthotopic transplantation of colon cancer cell lines in the colonic wall was successful in getting loco-regional metastases analogous to what is seen in human colon cancer.

Cluster 1: WP 1

**Technology development for bar coded RNAi screens
(NKI2)**

Workpackage completed, no new tasks.

Cluster 1: WP 2

**Generation of the murine RNAi vector library
(NKI2)**

Workpackage completed, no new tasks.

Cluster 1: WP 3

Development of ERMtag screens to identify pathway-specific cancer genes in mammalian cells

(NKI3)

Workpackage completed, no new tasks.

Cluster 1: WP 4

Development of Retrovirus-based living cell microarrays (IEO2)

The main goal of the project is the development of a robust and reliable technology for phenotype screening in array format in primary and cancer cells by using RNA interference approach.

As we reported in the previous annual report we have identified a novel biocompatible substrate that consists of an inorganic coating of glass slide of nanostructured TiO₂ film produced by a technology of Supersonic Cluster Beam Deposition (developed by Tethis S.r.l.), which can be properly functionalized to produce a stable array of retroviruses. This biomaterial mediates in situ localized infection of primary and cancer cells at high efficiency and allows, thanks to its high biocompatibility, to perform short and long term biological assays..

During 2006 we have performed preliminary tests on ns-TiO₂ to optimize the substrate with specific treatment (annealing at 400°C after film deposition) to obtain localized and efficient infection of GFP reporter genes in primary and cancer cells: in particular we tested the osteoblast cell line U2OS, the epithelial cell line MCF10A (Fig.1), the immortalized primary fibroblasts Tig3-Tert and human adult primary melanocytes (Fig.2), a very difficult model to be genetically transduced at good efficiency. We present here arrays of around 250-300 spots with two viruses localized in two different compartments (GFP for cytoplasmic localization and GFP-NPM for nucleolar localization) in order to further verify in situ localized overexpression in a complex array. We obtained high efficiency of localized infection for all the four cellular models tested.

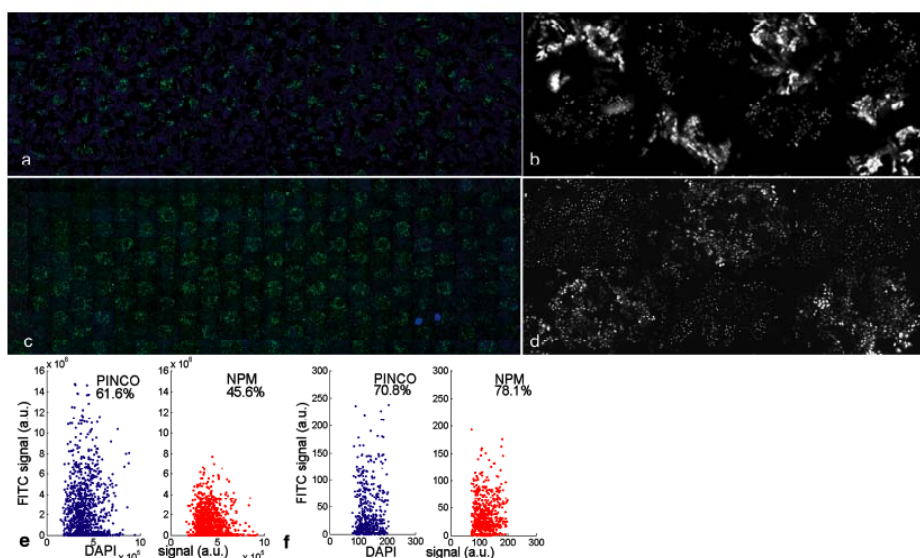
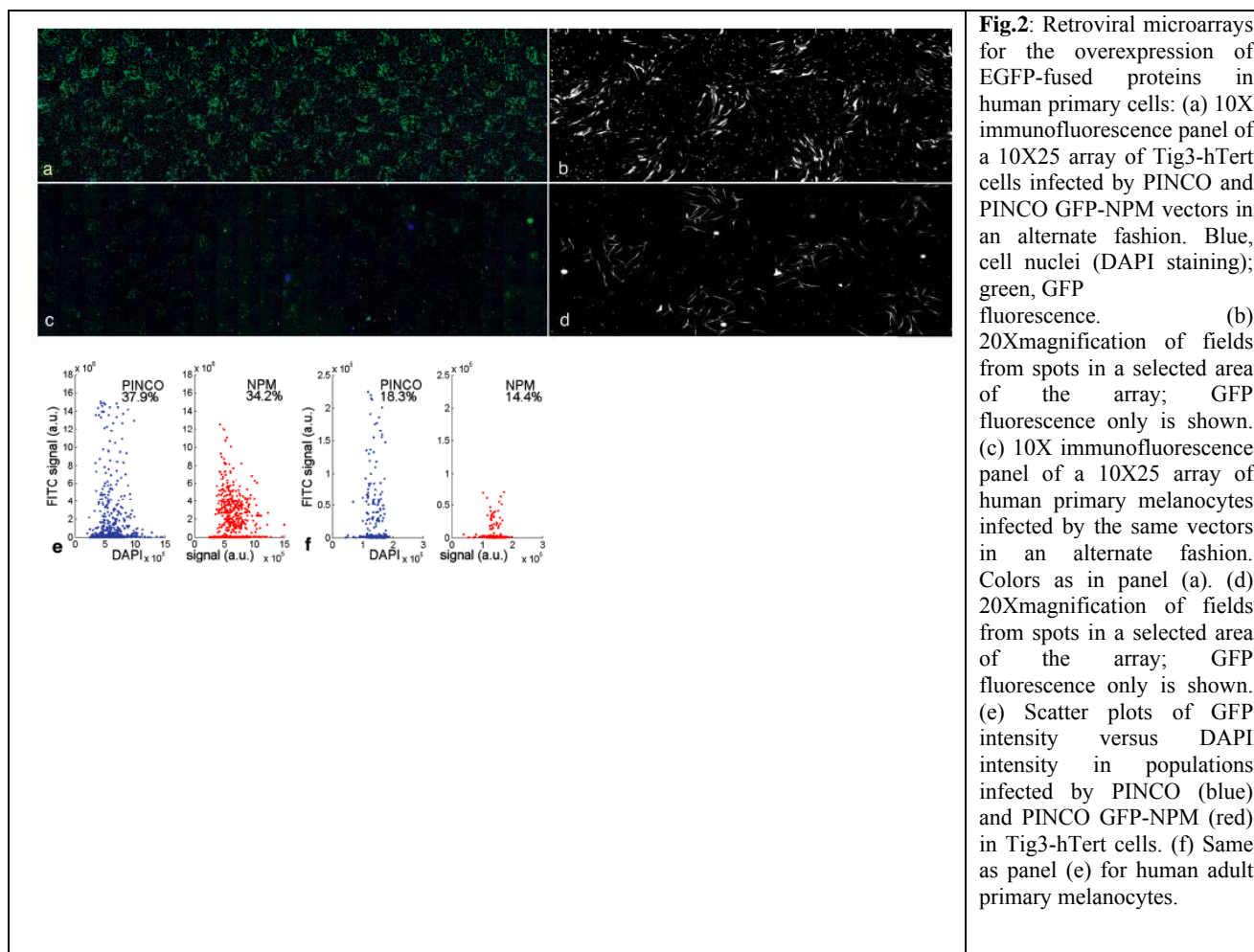
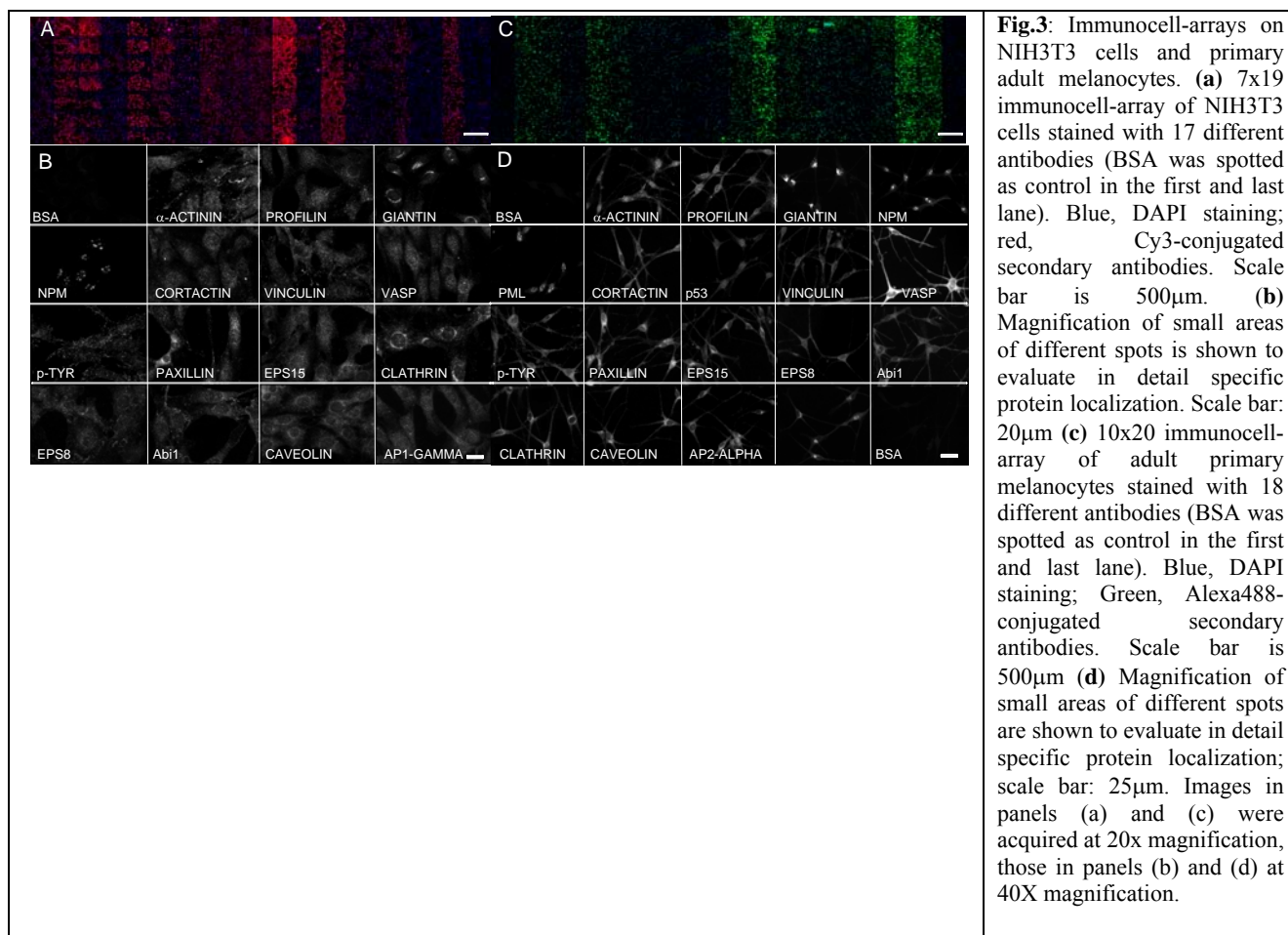


Fig.1: Retroviral microarrays for the overexpression of EGFP-fused proteins: (a) 10X immunofluorescence panel of a 9X25 array of MCF10A cells infected by PINCO and PINCO GFP-NPM vectors in an alternate fashion. Blue, cell nuclei (DAPI staining); green, GFP fluorescence. (b) 20 X magnification of fields from spots in a selected area of the array; GFP fluorescence only is shown. (c) 10X immunofluorescence panel of a 9X25 array of U2OS cells infected by the same vectors in an alternate fashion. Colors as in panel (a). (d) 20X magnification of fields from spots in a selected area of the U2OS array; GFP fluorescence only is shown. (e) Scatter plots of GFP intensity versus DAPI intensity in populations infected by PINCO (blue) and PINCO GFP-NPM (red) in MCF10A cells. The percentage of infected cells is reported. (f) Same as panel (e) for U2OS cells.



We have extensively tried to produce good quality DNA from a subset of genes of the kinase library and consequently high titer concentrated viral supernatant: we encountered different problems related to the genomic instability of such clones or the low yield of retroviral titre. For this reason we decided to delay the goal of preparing the “retroviral kinase chip” and concentrated our effort in the development of a technology of immunofluorescence analysis on glass slide to simultaneously analyze protein expression in spots. We have developed an “immunocell-array” of cells on chip where, upon cell plating, growing and selection, by spotting specific primary and secondary antibodies we can detect the localization and state of hundreds of proteins involved in specific signaling pathways; this technology could allow the simultaneous detection of protein down-regulation of hundreds of different RNAi clones in a single glass slide. We present an example of “immunocell-array” of NIH3T3 and adult primary melanocytes (Fig. 3) plated on glass slide and probed with different antibodies to analyse target localization. This novel technology represents the necessary complement to correctly perform cell-based assay by RNA interference on chip: with this approach hundreds of genes can be simultaneously detected for down-regulation efficiency directly on the target and obtain a phenotypic readout by any specific biological immunofluorescence assay.



Objectives (revised due to change of strategy):

- Optimization of primary and cancer cell infection on retroviral cell array on TiO₂ chip by GFP based retrovirus
- Development of a new technology of immunofluorescence on glass slide by antibody spotting on fixed cells for the simultaneous analysis of protein expression in different cellular models

Activity list of tasks, deliverables and milestones in the WP (months 1-42):

	No.	Task name	Status	Associated milestone	Milestone date
original	T 4.1	Optimize protocols for high throughput production and storage of retroviral supernatant.	Achieved	M 4.1	12
	T 4.2	Setting up conditions for viral supernatant concentration.	Achieved	M 4.1	18
	T 4.3	Establish efficient methods for viral particles spotting with automatic arrayer.	Achieved	M 4.2	18
	T 4.4	Establish methods for efficient immobilization of viral particles on glass slides.	Achieved	M 4.3	18
	T 4.5	Optimize protocols for high efficiency of infection for primary cells.	Achieved	M 4.4	12

Third Annual Periodic Activity Report (2006)

Update 13-30	T 4.6	Produce high titre viral supernatant from the pRETROSUPER kinase library.	Delayed for technical problems	M 4.5	18
	T 4.7	Analysis of titre and normalization of the supernatants	Delayed for technical problems	M 4.6	24
	T 4.8	Production of normalized retrovirus chip of the kinase library	Not started yet	M 4.7	27
	T 4.9	Analysis of efficiency of RNAi through inverse infection on primary and cancer cells by immunofluorescence analysis of selected known kinases	Delayed for technical problems	M 4.8	30
Update 25-42	T 4.10	Optimization of substrate properties and patterning on glass slide for high efficiency of infection in primary and cancer cells	Achieved	M 4.9	48
	T 4.11	Array of pRetrosuper vectors of kinase library with analysis of RNA interference efficiency on chip	Not started yet	M4.10	48
	T 4.12	Set up of an immunofluorescence assay on chip for detection of protein expression and localization in cells upon RNAi	Achieved	M4.11	42

Cluster 1: WP 5

Activity D. Lentiviral based pathway regulation in *in vivo* mouse models (NK11)

The main objective of WP05 was to generate (inducible) lentiviral gene transfer systems and methods that can be effectively applied to introduce regulatable genes and shRNAs in mice. These vectors encode i(mproved)Cre recombinase to allow switching of oncogenes and tumor suppressor genes in conditional mouse models which will lead to tumor formation. Within the same vectors, a Tet-inducible shRNA is encoded which permits us to study, in the presence or absence of doxycyclin, tumor development under controlled conditions.

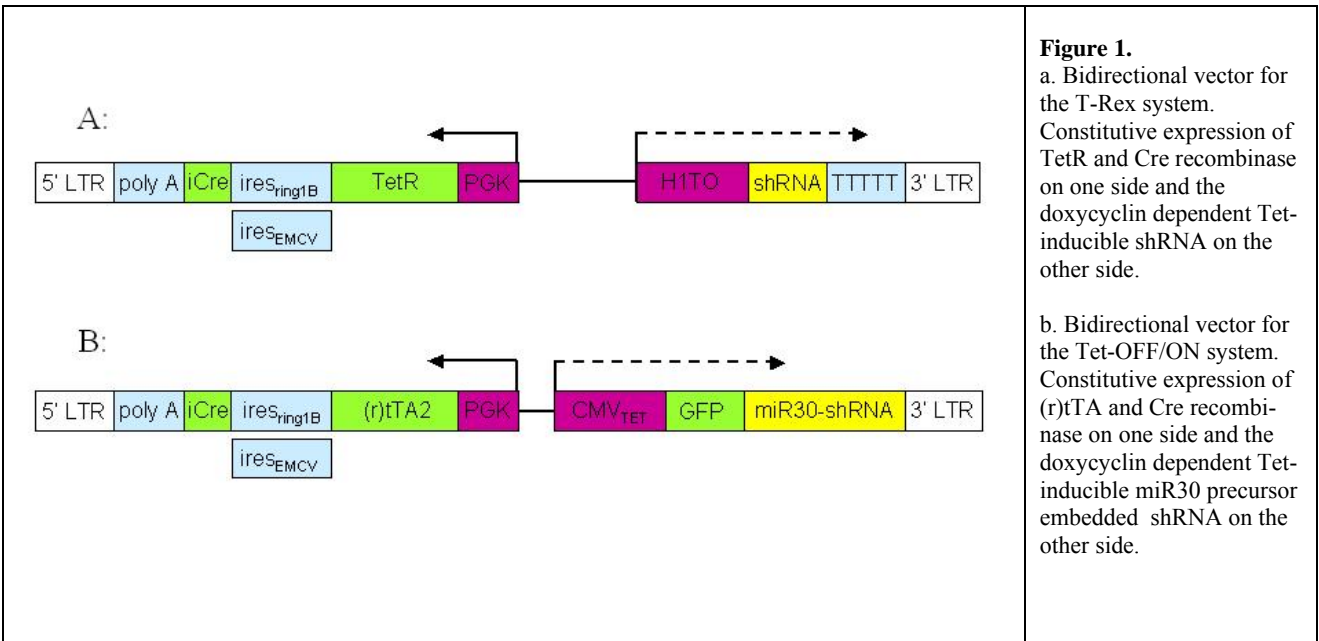
During the first years of the project, the desired lentiviral vectors encoding iCre recombinase as well as a Tet-inducible shRNA using the T-REx, Tet-OFF or Tet-ON system were constructed. However, unfortunately not all components of the generated vectors worked properly.

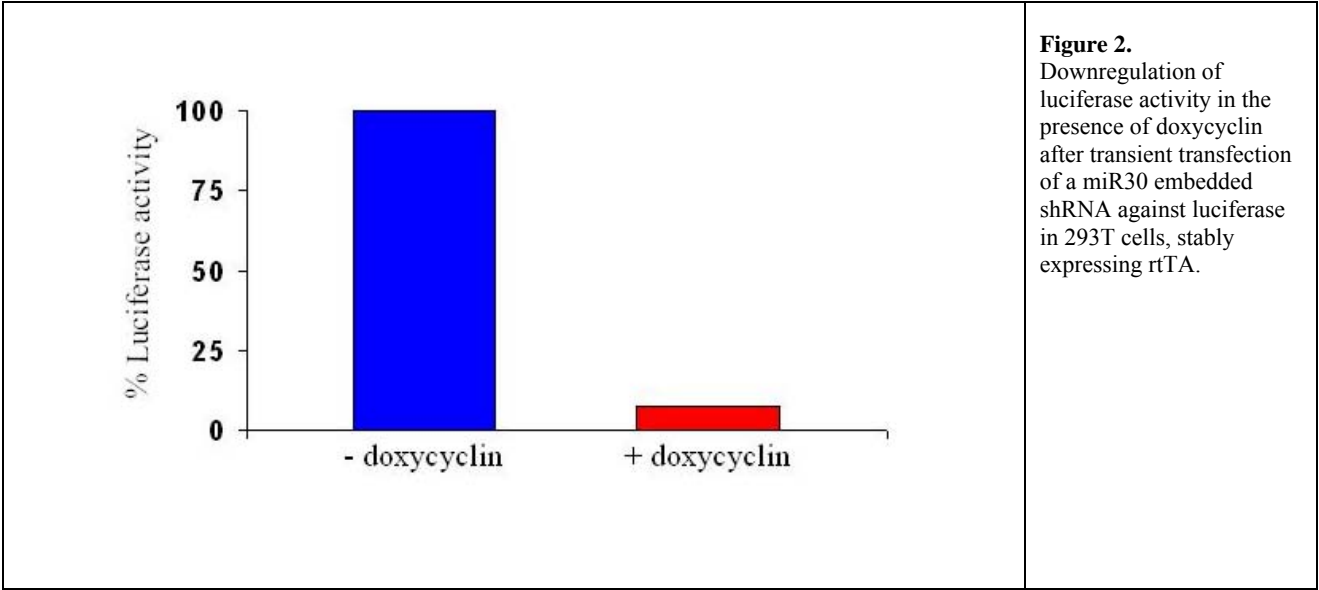
Therefore, last year we optimized the generated vectors (Figure 1). In case of the T-REx system, we are now able to regulate tightly the H1TO-shRNA by doxycyclin. However, expression levels of TetR protein are too low to interfere completely with the H1TO promoter in the absence of doxycyclin. In order to solve this problem, we will test whether addition of an enhancer might increase TetR protein expression to the desirable level.

In case of the Tet-OFF and Tet-ON systems, doxycyclin dependent inducibility of a shRNA was achieved using the latest technique of RNA interference, the microRNA embedded shRNA.

For this, a Tet-responsive minimal CMV promoter regulates a shRNA which is embedded within the miR30 precursor. *In vitro* data using a miR30-shRNA against luciferase shows tightly regulatable and efficient knock down of luciferase (Figure 2).

All together, the progress we made during the last year concerning the optimization of the functional components of the lentiviral vectors has been successful and therefore *in vivo* validation of the vectors will be the major activity of next year.

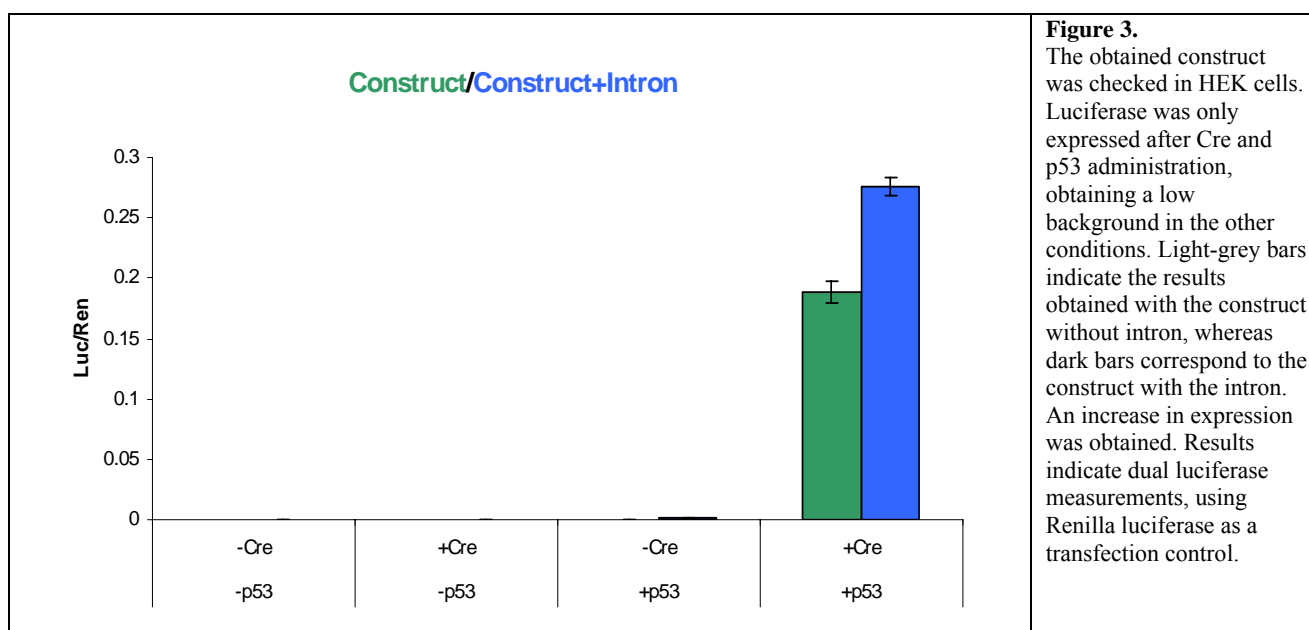




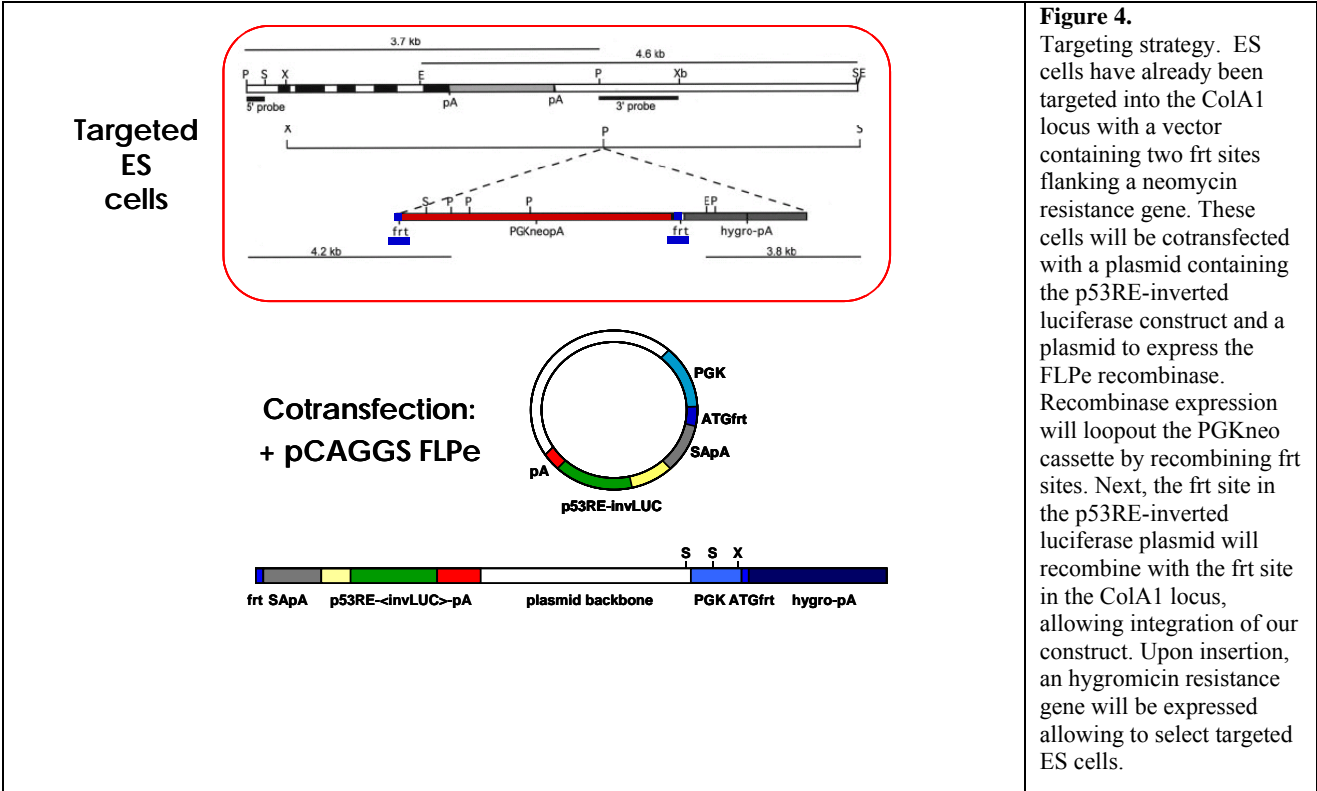
The second goal was to generate pathway reporters that would allow monitoring of pathway activation *in vivo* in mice using non-invasive imaging methods. We have focused on p53 reporters. A construct where a reporter gene expression is driven by a p53 responsive element has been made. Among the different p53-responsive elements that were tested, our final decision was for an artificial binding site, made of 14 repeats of the consensus binding sequence of p53. The selected reporter gene was the synthetic *Photinus pyralis* luciferase gene, which has been codon-optimized for mammalian expression and for reduced anomalous expression.

We have chosen a strategy such that luciferase expression by p53 activation will remain silent in all tissues except in those in which we are interested. To achieve this, the inverted luciferase gene is flanked by two mutated lox sites (lox66 and lox71). Following tissue-specific Cre expression, the lox sites will recombine and the luciferase gene will be in the correct orientation. The use of mutated lox sites will avoid recurrent recombination of the lox sites and, therefore, undesired inversion of the reporter gene.

Downstream the p53-responsive element, we have added a chimeric intron composed of the 5'-donor site from the first intron of the human β -globin gene and the branch and 3'-acceptor site from the intron that is between the leader and the body of an immunoglobulin gene heavy chain variable region. This intron will allow increased expression of luciferase *in vivo*. At this moment, we already have this final construct and it has been checked *in vitro*, showing that it works only when Cre is expressed and p53 is active (Figure 3).



In order to generate the p53 reporter transgenic mouse, we will knock-in the construct into the ColA1 locus taking advantage of the flp-in strategy developed recently in the lab of Rudolf Jaenisch (Beard *et al.*, Genesis, 2006) (See Figure 4 for an overview of the strategy). This system will allow us to avoid random integration of the construct into the genome, controlling the place where it has been inserted. It will also prevent silencing due to repeated-tandem integration of the construct and DNA rearrangements caused by lox site recombination other than the one expected. At this moment our construct has been cloned into the targeting vector to transfect ES cells and select for correctly targeted cells. ES cells with the integrated construct will be selected with hygromycin and Southern blot will be performed to check that the construct has been correctly targeted. Mice will be derived by tetraploid embryo complementation from ES cells carrying the construct.



Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 05.1	Generation of a versatile in vivo validation system for cancer targets using lentiviral vectors for introducing tet-inducible cDNAs and RNAi constructs into either somatic cells or the germ line of mice.	In progress	M 05.1	21
	T 05.2	Optimization of infection conditions for VSV-G pseudotyped HIV-based vectors for the efficient introduction of expression construct in pre-implantation embryo's. Initially, we set up procedures for Lentiviral transgenesis using pre-implantation embryos.	Achieved	M 05. 2	21
	T 05.3	Generation of a generally expressed tetracycline repressible transcriptional activator/repressor mouse line.	Achieved	M 05. 3	18
Update 12-30	T 05.4	Generation of reporter mice for p53. A minimal promoter linked to a tandem set of p53 binding sites will be used to drive expression of a dual and triple reporter constructs	In progress	M 05.4	30

Cluster 1: WP 6

Establishment of a mouse xenograft platform to validate putative cancer therapy targets (ISREC2)

With this technology section of the program, we intended to create a versatile platform for validation of cancer-related genes in *in vivo* mouse model systems.

The original project was based on the induction of dominant negative variants to block activated pathways in cancer cells, such as the Wnt pathway in colon carcinoma cells. The potential to suppress target gene expression with shRNA-based vectors convinced us to use this approach for *in vivo* validation of oncogenes. Difficulties with CRE-based irreversible induction systems due to multiple integrations, made us switch to use Tet regulatable systems. To make this strategy versatile, and perhaps even feasible for primary tumor-derived cells, we have chosen to use lentiviral transduction.

In the first year we have therefore introduced the tools and protocols for the lentiviral work. We acquired the expertise to manipulate these recombination-prone plasmids and obtained the conditions to produce high titer concentrated lentivirus stocks. As a first strategy we have chosen the LS174T colon carcinoma cell line as a model system. It has been shown that suppression of β -catenin expression or, alternatively, forced expression of a dominant negative variant of TCF4 leads to growth arrest and differentiation of these cells. The two-vector system for DOX-regulatable induction of shRNA described by D. Trono and colleagues, was introduced to inducibly suppress β -catenin expression. LS174T cells were infected with the vector that expresses the TetR/KRAB regulator fusion protein. A single clone was selected that expressed the regulator at low levels (as assessed by the presence of IRES-dsRed). Multiple infection rounds finally resulted in sufficient knockdown to induce the described phenotype in these cells. A control infection using an shRNA targeting mouse Pygopus2 was carried out in parallel. For a control shRNA which targeted EGFP, single copy insertions, and hence low MOIs, showed up to 95% knockdown after doxycyclin induction. As the level of luciferase expression obtained by stable transfection was too low for imaging, we performed an additional infection round with pLVCL to increase the luciferase activity.

In the second year, we introduced non-invasive luciferase-based imaging and the subcutaneous grafting of tumor cells on mice. The LS3T5 clones expressing a control shRNA and the β -catenin shRNA were grafted on the flanks of NOD/SCID mice. The mice were either treated with 1mg/ml doxycyclin in the drinking water, or were left untreated. Surprisingly, tumor outgrowth was similar for β -catenin knockdown tumors as compared to the controls. Histological assessment showed that tumor morphology of the knockdown tumors showed large holes and an overall low cellular density. The knockdown of β -catenin was confirmed by IHC. This analysis revealed also some islands of cells where the protein was still left. These areas coincide with Ki67-positive, highly proliferative areas, where EGFP expression (co-regulated with the shRNA by doxycyclin) was low. This can be explained by insufficient doxycyclin penetrance in the tissues, or cell clones that did not express sufficient shRNA. Alcian blue staining showed that the knockdown tumors expressed massive amounts of mucus, clearly indicative of differentiation.

IHC analysis for E-cadherin and ZO-1 showed no difference between the β -catenin knockdown vs. the control tumors, suggesting similar adhesive properties. The outgrowth of the tumors can be explained in different ways. First, luciferase imaging may not be adequate to assess cellular density. Secondly, Ki67 staining showed still proliferation in the highly differentiated areas. To address these issues, we generated a lentiviral vector where the induction of the shRNA is coupled to the induction of luciferase (as opposed to EGFP in the pLVTHm series by Trono; see figure 6.3.b.). As titers for these viruses were lower, we had difficulties to obtain cell clones with the same doxycyclin-induced phenotype as before. Figure 6.1 shows the difference in luciferase activity between doxycyclin-treated cells that inducibly suppress β -catenin

expression and control cells. Although the system is clearly leaky, it is likely going to indicate well whether proliferation is impaired in the knockdown cells.

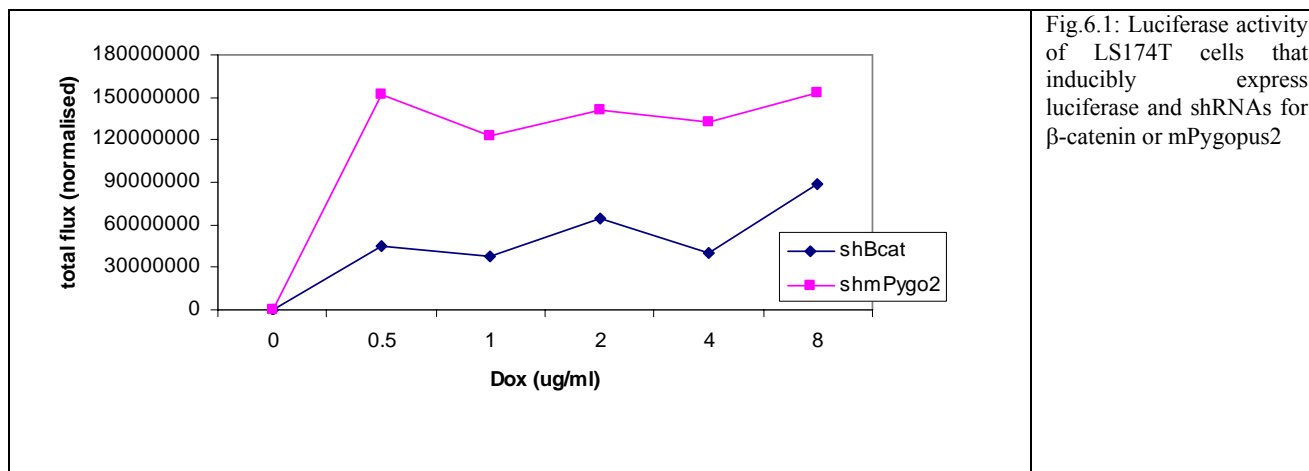
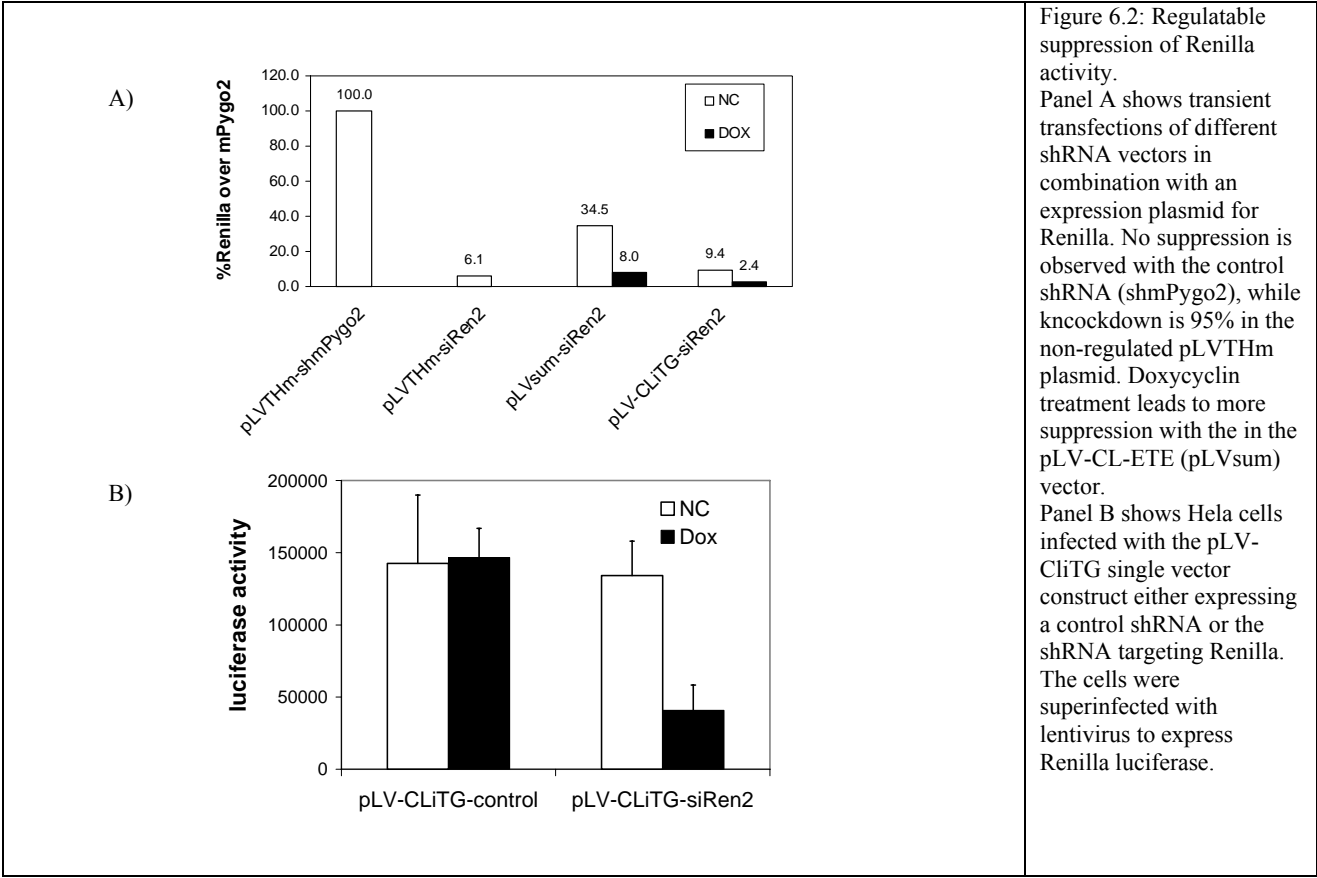


Fig.6.1: Luciferase activity of LS174T cells that inducibly express luciferase and shRNAs for β -catenin or mPygopus2

Throughout the project we have constructed and assessed different lentiviral vectors that contain all the components for luciferase expression and regulated shRNA expression. Using separate vectors for the different components will create a bias, which will only make the analysis of the *in vivo* knockdown effect more complicated. Multiple cloning steps, with the idea to keep the components exchangeable and compatible with the original pLVTHm series plasmids, in combination with unstable recombination-prone vectors, resulted in a serious delay in the construction of this vector.

Using an shRNA for Renilla for validation, the first series of dual promoter vectors (figure 6.3.a.: pLV-CL-PTE-siRen, pLV-CL-ETE-siRen) did not provide inducible Renilla knockdown. All components were tested separately in transient experiments and were shown to be functional. Although titers were low, we could obtain sufficient infection and low to very low nuclear expression of the TetR-EGFP regulator for pLV-CL-ETE-siRen and pLV-CL-PTE-siRen, respectively. The shRNA for Renilla did not deliver the expected knockdown in a non-inducible vector.



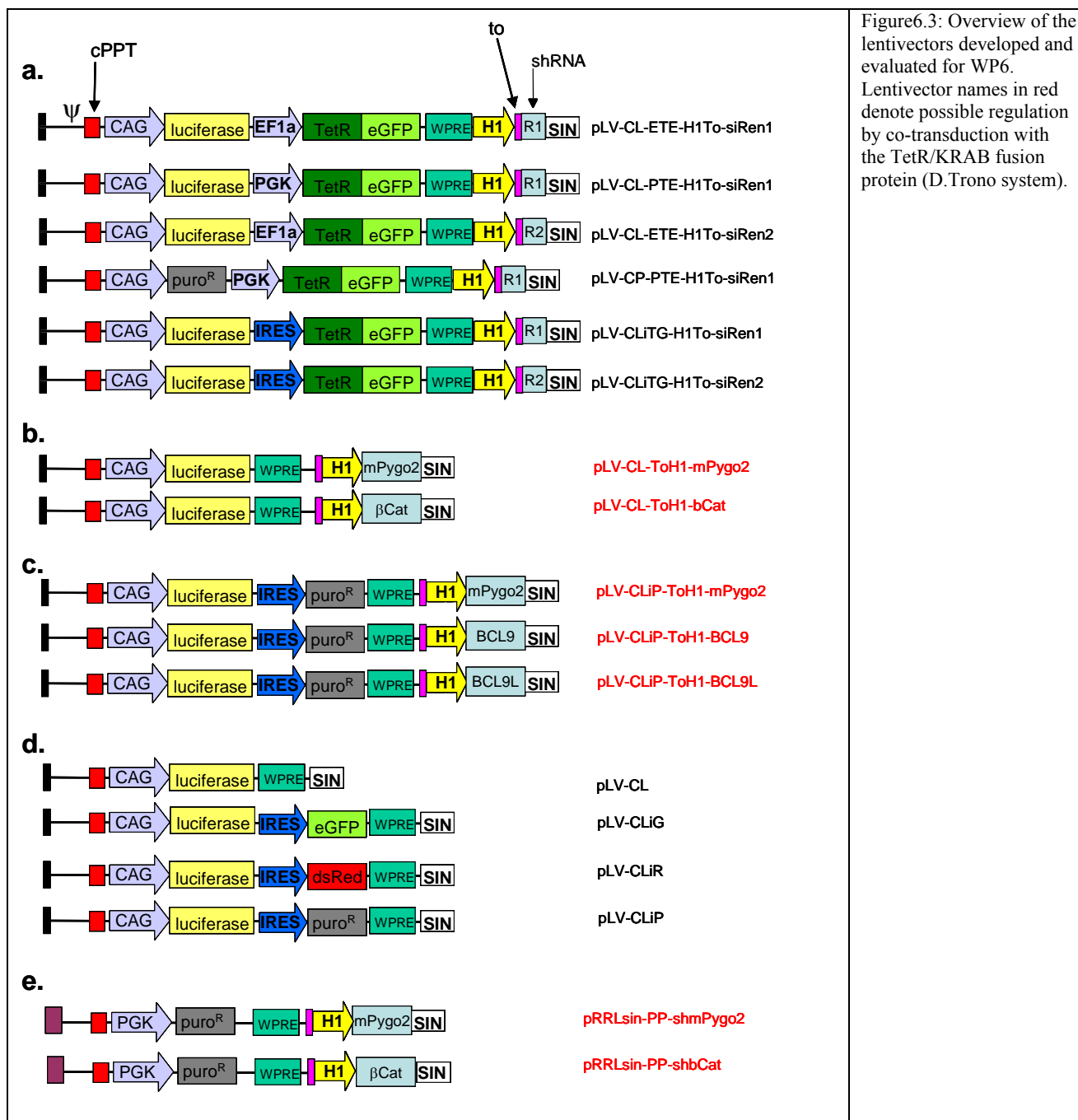


Figure6.3: Overview of the lentivectors developed and evaluated for WP6. Lentivector names in red denote possible regulation by co-transduction with the TetR/KRAB fusion protein (D.Trono system).

Due to time constraints we were unable to generate a stable cell clone expressing humanized Renilla. We have therefore infected Hela cells with the different single vector constructs and assessed the EGFP expression of these cells. We observed high infection efficiencies throughout all experiments. These cells were then superinfected with a lentivector expressing hRenilla. For these experiments, doxycyclin treatment resulted in additional knockdown effect with the pLV-CLiTG-siRen2 vector. These experiments should be repeated using a cell clone stably expressing hRenilla (Figure 6.2).

Additional shRNAs should be used to validate the utility of the vectors. To obtain knockdown with single copy integrations, it is required to have highly efficient shRNAs. As the algorithms do not permit to identify these shRNAs easily and as the validation proved to be more difficult than anticipated due to problems with Sybr Green quantitative RT-PCR, we have opted to change the strategy for validation of BCL9/Legless and Pygopus. We have therefore constructed lentivectors that express luciferase in combination with the IRES-

Third Annual Periodic Activity Report (2006)

Puro^R selection cassette. These also contain shRNAs for BCL9 or BCL9L, or the mPygo2 control (Fig. 6.3.c), respectively. SW480 and Co115 cells were infected with these vectors and afterwards selected with puromycin. The knockdown efficiency is currently being evaluated. Subcutaneous or orthotopic grafting will show whether there are any effects on tumor biology.

During the development of this single vector approach, we have generated a set of vectors that are of use for different purposes. Figure 6.3.d provides an overview of these lentivectors. A simplified vector based on the backbone developed by L. Naldini with puromycin selection and shRNA expression was also generated (Fig. 6.3.e)

Orthotopic grafting

In 2006 we have tested different parameters to establish orthotopic grafting with cell suspensions in the colon wall. The list below gives an overview of these parameters:

- Colon carcinoma cell lines:
All cell lines were infected with lentivirus to express luciferase, sometimes in combination with EGFP
CT26 (mouse Balb/C derived, can be used syngeneically)
LS174T
Co115
HT29
HCT116
SW480
- Cell numbers: 100K-500K
- Injection medium (e.g. use of mouse serum)
- Fibrin clot: using a fibrinogen-thrombin mixture the cells were immobilized after injection
- Location: upper and middle colon, caecum
- Mouse strains: NOD/SCID, nude
- Co-grafts with fibroblasts
- Lentivectors: pLVCL vs. puromycin selectable pLV-CLiP

Upon injection of CT26 cells, we obtained primary tumors with dissemination into lymph nodes along the intestinal system, all the way to pancreas and portal vein in the liver. This reflects what happens in patients suffering from colon cancer. However, as this is an extremely aggressive mouse cell line (5000 cells generate a lethal tumor burden in 20 days), we would prefer to use a less aggressive human cell line.

For all the tested human cells, in different conditions, we hardly found substantial primary tumors at the injection site. For most cell lines we could, however, observe lymph node tumors, ascitic growth and sometimes caecal tumors. Whether this is due to dispersal under the mesenterium surrounding the intestinal system or to cell leakage from the injection site is hard to address. We concluded that the injection of cell suspensions in the colon or caecum wall will likely not give reproducible tumor engraftment. This approach is therefore not suitable for our applications. Alternatives for the injection of cell suspensions can be the use of matrigel, cell clumps (e.g. by growth on non-adherent plastic), cells grown on porous beads, or the engraftment of tumor parts from subcutaneous tumors. To establish a good protocol for orthotopic grafting, more experiments and optimization steps are required. Due to these problems the use of primary cells was postponed until the protocol for engraftment with cell lines was established. The protocols for generation and infection of primary colon cancer cells were introduced.

Objectives (revised due to change of strategy)

- Establish a human/NOD/SCID mouse tumour xenograft model to validate Lgs as a potential therapeutic target in colon cancer.
- Optimize this model to assess the impact on tumour growth and morphology of any gene of interest upon its induction in an established human tumour xenograft.

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 30):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 6.1	Design and validate functional siRNA's for Legless1 and 2, Pygopus1 and 2, TCF4 and β -catenin	in progress	M 6.1	18
	T 6.2	Select LS174T cell line constitutively expressing luciferase and containing an inducible siRNA directed against Legless, Pygopus, TCF4 and β -catenin	in progress	M 6.1	18
	T 6.3	Establish and optimize conditions for reproducible engraftment of this cell line in a nude or NOD/SCID mouse model system: compare different clones with regard to engraftment, histology, and efficacy of gene of interest induction within the tumour	in progress	M 6.2	21
	T 6.4	Introduce luciferase-based non-invasive imaging of human tumour progression in mice. Subject tumour grafts to histological assessment of cell proliferation, apoptosis and cell differentiation upon induction of the siRNA cassette	in progress	M 6.3	24
	T 6.5	Construction and evaluation of a single vector lentiviral system containing a TetR-regulated siRNA cassette, and expression cassettes for TetR and luciferase	in progress	M 6.4	18
	T 6.6	Evaluate different approaches for engraftment of primary tumour material	not started yet	M 6.5	30
Update 12-30	T 6.1	Design and validate functional siRNA's for Legless1 and 2, Pygopus1 and 2, TCF4 and β -catenin	Partially	M 6.1	18
	T 6.2	Select LS174T cell line constitutively expressing luciferase and containing an inducible siRNA directed against Legless, Pygopus, TCF4 and β -catenin	Partially	M 6.1	18
	T 6.3	Establish and optimize conditions for reproducible engraftment of this cell line in a nude or NOD/SCID mouse model system: compare different clones with regard to engraftment, histology, and efficacy of gene of interest induction within the tumour	Achieved	M 6.2	21
	T 6.4	Introduce luciferase-based non-invasive imaging of human tumour progression in mice. Subject tumour grafts to histological assessment of cell proliferation, apoptosis and cell differentiation upon induction of the siRNA cassette	Achieved	M 6.3	24
	T 6.5	Construction and evaluation of a single vector lentiviral system containing a TetR-regulated siRNA cassette, and expression cassettes for TetR and luciferase	In Progress	M 6.4	18
	T 6.6	Evaluate different approaches for engraftment of primary tumour material	In progress	M 6.5	30
Update 25-42	T 6.1	Design and validate functional siRNA's for Legless1 and 2, Pygopus1 and 2, TCF4 and β -catenin	Partially	M 6.1	18
	T 6.2	Select LS174T cell line constitutively expressing luciferase and containing an inducible siRNA directed against Legless, Pygopus, TCF4 and β -catenin	Partially	M 6.1	18
	T 6.3	Establish and optimize conditions for reproducible engraftment of this cell line in a nude or NOD/SCID mouse model system: compare different clones with regard to engraftment, histology, and efficacy of gene of interest induction within the tumour	Achieved	M 6.2	21
	T 6.4	Introduce luciferase-based non-invasive imaging of human tumour progression in mice. Subject tumour grafts to histological assessment of cell proliferation, apoptosis and cell differentiation upon induction of the siRNA cassette	Achieved	M 6.3	24
	T 6.5	Construction and evaluation of a single vector lentiviral system containing a TetR-regulated siRNA cassette, and expression cassettes for TetR and luciferase	Achieved	M 6.4	18
	T 6.6	Evaluate different approaches for engraftment of primary tumour material	In progress	M 6.5	30

SUMMARY of research activities during 2006

Cluster 2 (WPs 7-13): DNA damage and p53 (UKBH, CNIO1 (coordinator), BICR, NKI2, IEO2)

The main goal of cluster 2 is to identify novel cancer-relevant regulators and effectors of the DNA damage response using shRNA-based screening strategies. Initial troubles with the setup of useful screening assays and problems with the integration of activities within the cluster have been successfully overcome during 2005.

During 2006, most of the proposed screening approaches have been terminated successfully, positive hits been validated and put forward to functional analysis. In particular, UKBH identified a novel component (NEK11) involved in the regulation of the DNA damage response in higher eukaryotes and is currently characterizing three other promising hits implicated in G2/M damage checkpoint regulation. Optimization and validation of a sophisticated high throughput screening platform for telomere length measurements, developed by CNIO1, has allowed to pick up putative regulators of telomere length that are currently being validated and characterized. A close collaboration between BICR and CNIO2 has come up with a number of novel players in the complex and still poorly understood regulation of the p53 pathway, among them CRMP2, RPS6KA4, RUNDC1 and GAS41, which have been functionally validated as novel antagonists of p53 function (Figure 1). In addition, a novel role for ubiquitin specific protease enzymes for the regulation of p53 and the p53 target PUMA is emerging from screens performed by BICR. Finally, the re-design of NKI's synthetic lethal screen has been successfully terminated, and first results with synthetic siRNAs targeting the human kinome have yielded promising candidates for synthetic lethal interactions with p53.

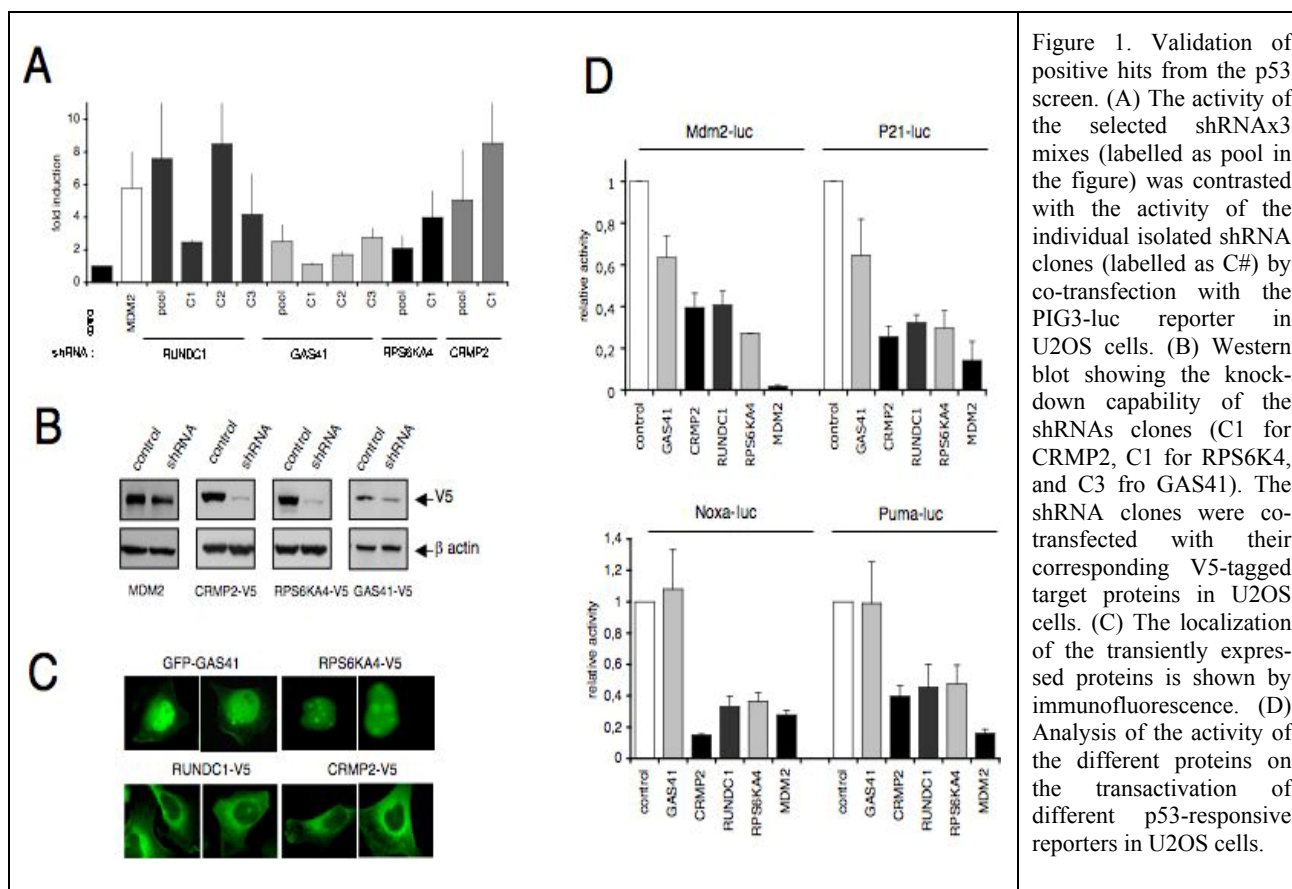
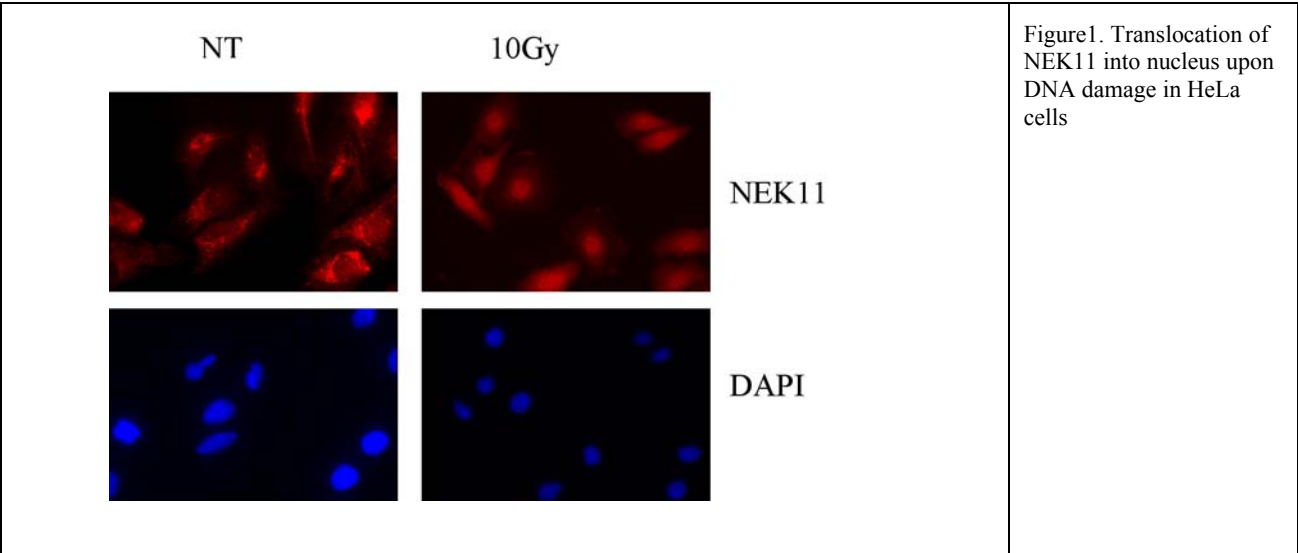


Figure 1. Validation of positive hits from the p53 screen. (A) The activity of the selected shRNAx3 mixes (labelled as pool in the figure) was contrasted with the activity of the individual isolated shRNA clones (labelled as C#) by co-transfection with the PIG3-luc reporter in U2OS cells. (B) Western blot showing the knock-down capability of the shRNAs clones (C1 for CRMP2, C1 for RPS6KA4, and C3 for GAS41). The shRNA clones were co-transfected with their corresponding V5-tagged target proteins in U2OS cells. (C) The localization of the transiently expressed proteins is shown by immunofluorescence. (D) Analysis of the activity of the different proteins on the transactivation of different p53-responsive reporters in U2OS cells.

Cluster 2: WP 7
Targeting the ATM-ATR/CHK1-CHK2 pathway using RNAi and ERMtag-based screens (UKBH)

As was reported previously, we have screened for genes whose inactivation leads to bypass of the G2/M checkpoint in response to ionizing radiation. Subsequently we have validated the by using different RNAi's. Four genes were selected for further characterization: the NEK11 and PIP5K2A kinases, the PDPR pyruvate dehydrogenase phosphatase regulatory subunit and the deubiquitinating enzyme CYCLD.

During 2006 we have focused on the characterization of the role the NEK11 kinase in the G2/M checkpoint. We have generated reagents required to study the kinase: mammalian cell expression constructs, baculovirus expressed recombinant wild type and the kinase dead variants of NEK11, and rabbit polyclonal antibodies. We have characterized in detail the loss-of-function phenotype of NEK11. Using polyclonal antibodies to NEK11 we have shown that NEK11 is localized to mitochondria, nuclear envelope and nuclear bodies. Upon DNA damage NEK 11 is gradually released from the mitochondria and translocated into the nucleus (Fig. 1).



Our results indicate that the CDC25A phosphatase is a main substrate of NEK11 in the DNA damage signalling pathway. NEK11 can phosphorylate both in vivo and in vitro the S82S88 phosphodegron motif of CDC25A. The S82S88 phosphodegron motif is required for the binding of the F-box protein beta TRCP and for the ubiquitylation of CDC25A (Figure 2A). Consequently, loss of NEK11 prevents proteasomal degradation of CDC25A in both unperturbed cells and upon DNA damage (Figure 2B). Moreover, in vitro phosphorylation of the CDC25A phosphodegron by NEK11 is dramatically increased upon phosphorylation of serine 76 by CHK1. These biochemical data suggest that CHK1 and NEK11 cooperate to induce phosphorylation-dependent polyubiquitination of CDC25A.

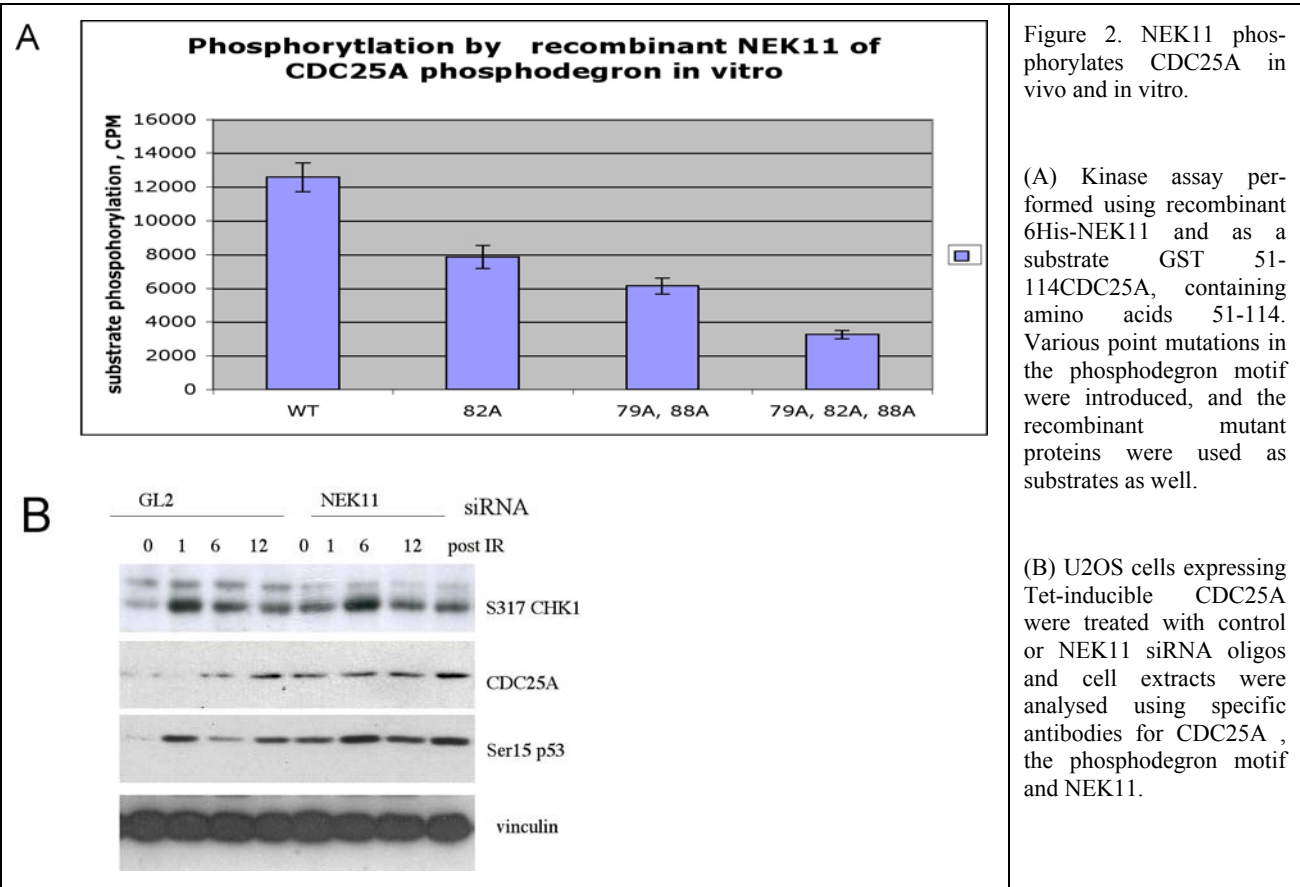


Figure 2. NEK11 phosphorylates CDC25A in vivo and in vitro.

(A) Kinase assay performed using recombinant 6His-NEK11 and as a substrate GST 51-114CDC25A, containing amino acids 51-114. Various point mutations in the phosphodegron motif were introduced, and the recombinant mutant proteins were used as substrates as well.

(B) U2OS cells expressing Tet-inducible CDC25A were treated with control or NEK11 siRNA oligos and cell extracts were analysed using specific antibodies for CDC25A, the phosphodegron motif and NEK11.

To understand the requirement of NEK11 in regulating the stability of CDC25A, we are currently putting efforts on understanding the mechanisms regulating NEK11 kinase activity. NEK11 kinase activity is activated by ionizing radiation and UV treatment. We are investigating the upstream pathways linking the DNA damage response to NEK11 activation. Preliminary results indicate that the activity and stability of NEK11 depend on CHK1. To extend these results, we are testing if NEK11 is a direct substrate for CHK1 and in addition we are mapping the CHK1 phosphorylation sites of NEK11.

To understand the regulation of NEK11 activity, we are purifying NEK11-associated proteins. So far, our approaches using ectopically expressed epitope-tagged NEK11 have not been successful, since overexpressed NEK11 is localized mainly to the cytoplasm and mitochondria and the localization did not change upon DNA damage. We are now testing the possibility to purify NEK11-associated proteins using immunopurification of endogenous NEK11.

Finally, we plan to test for alterations in NEK11 expression in primary human tumours using tissue microarrays (TMA) containing samples from primary human tumours. Our results suggest that NEK11 is a potential tumour suppressor gene whose inactivation leads to genomic instability and the development of cancer.

In summary, we expect that the results of this WP will lead to the identification of at least one novel component (NEK11) involved in the regulation of the DNA damage response in higher eukaryotes. Moreover, if time allows we will further characterize the 3 other genes previously isolated and validated, and we will perform another genome-wide screening for regulators of the G2/M checkpoint using high content screenings in 96 well format.

Third Annual Periodic Activity Report (2006)

Objectives (revised due to change of strategy)

- Identification and characterization of genes required for induction and maintenance of the G2/M checkpoint
- Study of the implication of G2/M checkpoint genes in human cancer

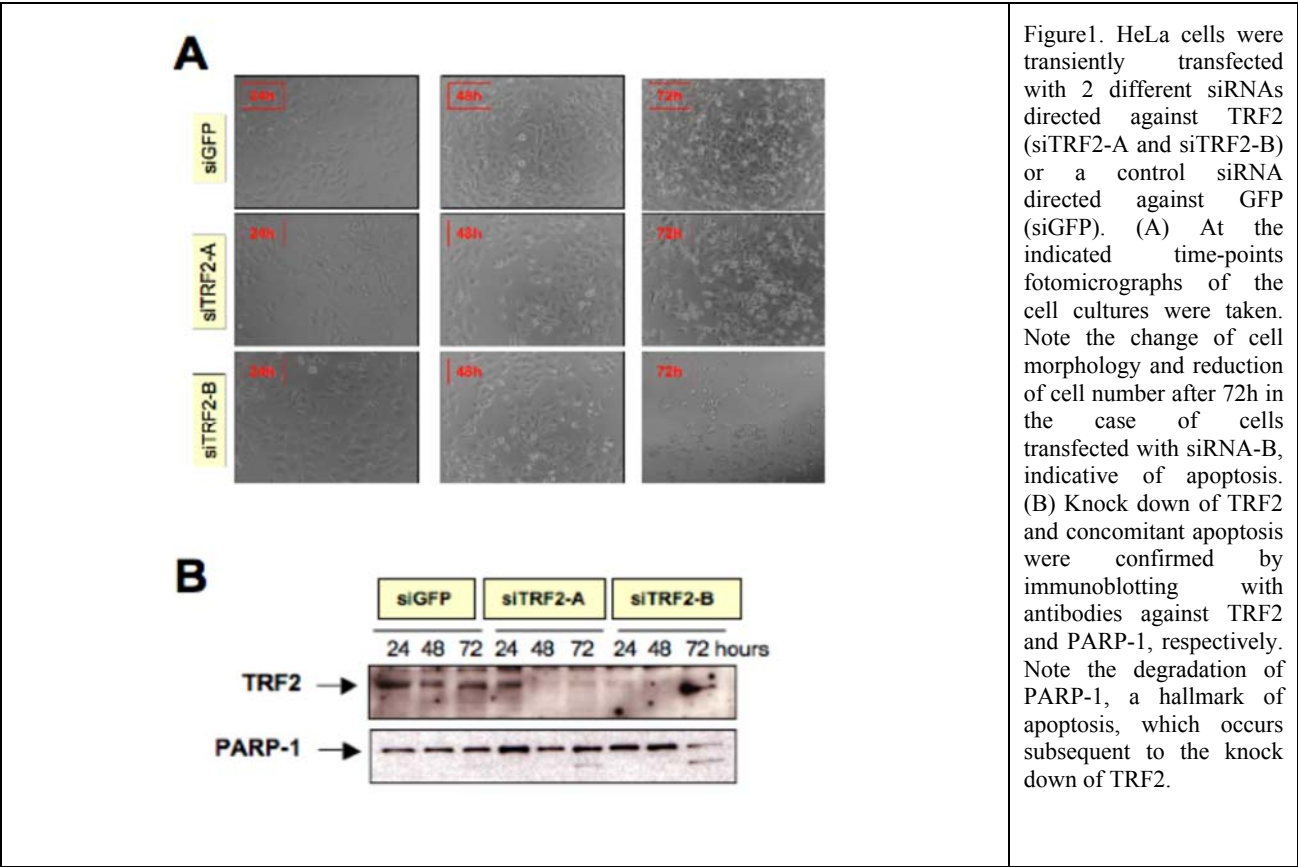
Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 7.1	Generation of human fibroblasts expressing the tet-repressor	Achieved	M 7.1	6
	T 7.2	Generation of stable cell lines (U2OS and diploid fibroblasts with the tet-repressor) expressing a CDC25-GFP fusion protein	Achieved	M 7.1	6
	T 7.3	Screening of tet-repressor cell lines expressing CDC25A-GFP using the ERM tag retroviral vector	Cancelled, alternative strategy	M 7.2	12
	T 7.4	Screening of the CDC25A-GFP cell lines using high-titer RNAi libraries	Cancelled, alternative strategy	M 7.3	12
	T 7.5	The expression of the identified genes in primary tumours will be analyzed by database analysis and screening of multiple tissue arrays by ISH	In progress	M 7.5	18
	T 7.6	The identified genes will be compared with the genes obtained in the screens in the other work packages	In progress	M 7.10	30
	T 7.7	Gene copy numbers for selected overexpressed or non-expressed genes will be determined by FISH	Not started yet	M 7.10	30
Update 12-30	T 7.8	Screening for genes that bypass the G2/M DNA damage checkpoint	Achieved	M 7.8	18
	T 7.9	Establishment of a reliable screening system for genes involved in the G2/M DNA damage checkpoint	Achieved	M 7.6-7	18
	T 7.10	Recovery and DNA sequencing of shRNAs isolated from the G2/M screen	Achieved	M 7.4	18
	T 7.11	Functional validation of the identified genes for their requirement in the initiation and maintenance of G2/M arrest after IR	Achieved and in progress	M7.9	30
	T 7.12	Characterization of the function of isolated genes in DNA damage response	In progress	M7.11	48
Update 25-42	T 7.13	Establishment and performance of HCS	Not started yet	M 7.12	36
	T 7.14	Isolation of protein complexes associated with a candidate gene product, analysis by MALDI TOF mass spectroscopy	In progress	M 7.11	48
	T 7.15	Identification of substrates of isolated kinases (phosphatases) in G2/M checkpoint pathway	Achieved and In progress	M7.11	48

Cluster 2: WP 8
Telomere damage response
(CNIO1)

Our aim was to develop an RNAi screening strategy, which allows the identification of downstream effectors of dysfunctional telomeres. Our screening strategy is based on the observation that knock-down of TRF2 in HeLa cells leads to a dramatic loss of cell viability within 72 hours. In this screen, it should be possible to pick up TRF2 knock-down cells that bypass cell death upon transfection with the human 8k NKI shRNA library 48 hours prior to cell death. shRNA inserts that rescue cells from apoptosis in this system are anticipated to yield genes that traduce telomere dysfunction into cell death.

During 2006, we have tested human cell lines transfected with a dominant negative form of hTRF2, as well as different shRNA and siRNA constructs directed against TRF2. The system that turned out to work best were HeLa cells transfected with an siRNA against TRF2. In this system, TRF2 levels were efficiently suppressed and, after 72 hours, cells entered into apoptosis as judged by a change of cell morphology and cleavage of PARP-1 (Figure 1). The key for a successful screen is to ensure that the background (cells that do not enter into or spontaneously escape apoptosis) is low enough to allow the reliable identification of positive clones (cells that escape apoptosis upon specific knock down of a pro-apoptotic gene). Unfortunately, this could not be achieved with this screening strategy. Due to technical problems in the set up of the screen and our unexpected advances in novel and very promising sub-projects within WP16 (epigenetic regulators of telomere function), we decided to abandon this approach and assign available resources to WP16.



Third Annual Periodic Activity Report (2006)

Objectives

- Cancelled due to technical problems in the set up of the screen and assignment of available resources to WP 16 (epigenetic regulators of telomere function)

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 30):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 8.1	Generation of mice and cells double knock-out for telomerase and telomere-binding proteins	Achieved	M 8.1	6
	T 8.2	Generation of RNAi retroviral vectors to knockdown telomere-binding proteins that are already known	Achieved	M 8.2	12
	T 8.3	Study of functional interactions between different activities at the telomere	Achieved	M 8.3	18
	T 8.4	Gene arrays analysis of both genetically modified mice and RNAi cellular systems to identify new targets for telomere dysfunction	Achieved	M 8.3	18
	T 8.5	Validation of the new targets by “real-time” PCR and RNAi	Achieved	M 8.4	18
	T 8.6	Screening of the different mouse models and cellular systems using high-titer RNAi libraries for identification of additional targets	Modified and replaced by T 8.9 due to change of strategy	M 8.5	18
	T 8.7	Generation of new mouse models	Stopped due to re-orientation of work plan at month 18 towards shRNA screenings	M 8.5	18
Update 12-30	T 8.8	Development of an RNAi screening system aimed at the identification of genes that bypass loss of cell viability caused by telomere dysfunction	Partially achieved	M 8.6-7	18
	T 8.9	shRNA screening for genes that bypass cell death induced by telomere dysfunction	Cancelled	M 8.8	24
	T 8.10	Functional validation of candidate genes	Cancelled	M 8.9-10	33-36

Cluster 2: WP 9

Role of telomerase in tumour promotion and metastasis (CNIO1)

With the aim in mind to contribute to INTACT with innovative shRNA screening technology, we have developed a high throughput telomere length quantification assay in 96-well format. This assay was designed to screen cultured cells for telomere length changes by measuring telomere fluorescence in a quantitative manner (Q-FISH) directly and simultaneously in 96-well tissue culture plates by robotized confocal scanning microscopy.

During 2006, this novel assay has been validated in human population studies with peripheral blood lymphocytes. The validation studies showed that the assay provides sufficient accuracy and sensitivity to reliably detect small changes in telomere length (Figure 1).

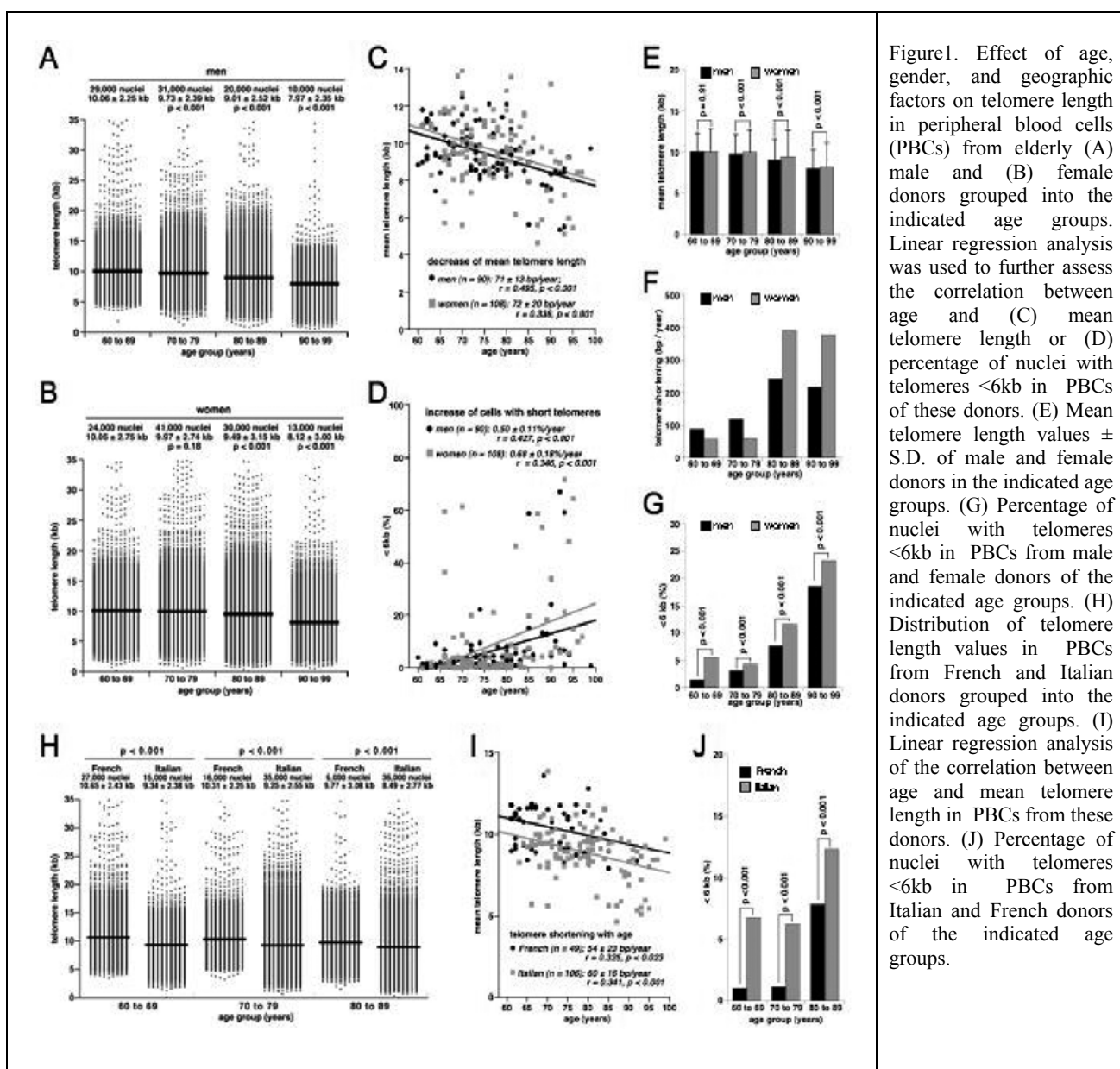


Figure 1. Effect of age, gender, and geographic factors on telomere length in peripheral blood cells (PBCs) from elderly (A) male and (B) female donors grouped into the indicated age groups. Linear regression analysis was used to further assess the correlation between age and (C) mean telomere length or (D) percentage of nuclei with telomeres <6kb in PBCs of these donors. (E) Mean telomere length values \pm S.D. of male and female donors in the indicated age groups. (G) Percentage of nuclei with telomeres <6kb in PBCs from male and female donors of the indicated age groups. (H) Distribution of telomere length values in PBCs from French and Italian donors grouped into the indicated age groups. (I) Linear regression analysis of the correlation between age and mean telomere length in PBCs from these donors. (J) Percentage of nuclei with telomeres <6kb in PBCs from Italian and French donors of the indicated age groups.

Third Annual Periodic Activity Report (2006)

A clone-by-clone screen of the entire 8k-NKI shRNA library in search for novel regulators of telomere length using this novel technology has been terminated both in HeLa cells with short (HeLa) and long (1211) telomeres. CNIO1 was able to pick up 16 and 6 shRNA inserts that induced a >1.5-fold increase of telomere length in HeLa and 1211 cells respectively. The positive clones are currently being validated by flow-FISH and/or Q-FISH analysis.

As a complementary approach to identify novel regulators of telomere length, CNIO1 has set up a screening system aimed at the identification of transcriptional regulators of telomerase. The screen is based on a HeLa cells transiently transfected with luciferase under control of a human Tert promoter spanning from –1375 bp to +80 bp. The screening system has been validated with an shRNA insert directed against myc, a known transcriptional activator of telomerase. The shRNA screen with the human 8k-shRNA library has not been performed yet.

Objective

- Identification of genes that regulate telomerase activity and telomere length

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 9.1	Generation of new telomerase transgenic mice with targeted Tert expression to adult tissues	Achieved	M 9.1	6
	T 9.2	Gene arrays analysis of genetically modified mice	Achieved	M 9.2	12
	T 9.3	Validation of the new targets	Achieved	M 9.3	18
	T 9.4	Screening of the different mouse models and cellular systems using high-titer RNAi libraries for identification of additional targets	Modified and updated to T 9.7	M 9.3	18
	T 9.5	Generation of new mouse models	Achieved	M 9.4	18
Update 12-30	T 9.6	Set-up of a high-throughput Q-FISH telomere length determination method	Achieved	M 9.5	15
	T 9.7	Set-up of a screening system aimed at the identification of transcriptional regulators of telomerase	Achieved	M 9.6	15
	T 9.8	Screening for genes that regulate telomere length	Achieved	M 9.7	24
	T 9.9	Screening for genes that regulate telomerase activity	Not started yet	M 9.7	24
	T 9.10	Functional validation of candidate genes	In progress	M 9.8	30

Cluster 2: WP10**Targeting the p53 pathway using RNAi and ERMtag-based screens (BICR (BICR/CNIO2))**

The main objective of WP10 is to identify regulators of the expression of p53-target genes, both at the overall level of p53 activation and as contributors to the differential transcriptional activity leading to the differential responses of cell cycle arrest vs. apoptosis. Previously, we had generated reporter constructs for p53 target genes p21 and Bax, and validated the activity of the promoters within these cells. In addition, we had developed a screening system based on transient transfection of U2OS cells using a PIG3-firefly-luciferase reporter and screened on a one-by-one basis the entire human 8k-shRNA library.

During 2006 we focused on using the reporter cells to screen for genes that regulate p53-dependent transcription. Our initial proposal was to use GFP as a promoter, so that we could screen pools of shRNA clones and identify functional clones by FACS. However, as describe in the 2005 report, this approach was unsuccessful, so we turned to the more fully validated reporter system using luciferase. Having established that the Bax-firefly-luc and PG13-renilla-luc reporters functioned as expected following transient assays, we set out to establish cell lines stable expressing the two reporter constructs, and testing their response to p53 activation. In light of the work of the previous reporting period, we opted to establish this reporter cell line using p53-inducible Saos-2 cells. Following co-transfection with Bax-firefly-luc and PG13-renilla-luc reporter constructs, and selection for puromycin resistance, 48 independent clones of cells were isolated, expanded and luciferase activity measured in the presence or absence of doxycycline (to activate p53 expression). Four of the 48 cell clones showed activation of both firefly and renilla luciferase (Table 1). Clones 35 and 38 were used for subsequent experiments.

<i>Saos-2 clone</i>	<i>Bax-firefly luciferase</i>	<i>PG13-renilla-luciferase</i>
# 20	12x	2x
# 35	6x	101x
# 25	4x	18x
# 38	36x	9x

Table 1. Fold activation of renilla and firefly luciferase activity in Saos-2 cells treated with doxycycline.

Our intention is to screen for genes whose expression can differentially modulate the activation of the two reporter constructs by p53. Since we have moved from the GFP reporter system to the luciferase system, a more laborious screening approach of infecting with the shRNA single gene library would be required. Since we do not yet have access to this library, we have we started by screening an oligonucleotide siRNA library purchased from Dharmacon, directed against DUBs. This library contained 96 pools of 4 siRNA oligonucleotides directed against individual DUBs. Following transfection with the siRNAs, p53 was induced by treatment with doxycycline and the firefly and renilla luciferase activity measured. The screen was carried out in duplicate, and siRNAs that reproducibly altered the activation of the Bax and PG13 promoters by p53 were identified. The results of the initial screens are shown in Table 2.

<i>Reduced activation of PG13 and Bax</i>	<i>Reduced activation of PG13 only</i>	<i>Reduced activation of Bax only</i>	<i>Increased activation of PG13 and Bax</i>	<i>Increased activation of PG13 only</i>	<i>Increased activation of Bax only</i>
9	4	0	2	1	2

Table 2. Number of different siRNA pools that affected the activation of PG13 and Bax reporters by p53

Based on the activity of the p53175P mutant, we had anticipated finding siRNAs that would differentially affect Bax, but not PG13 expression. However, in this initial screen we did not identify any siRNAs that fell into this category. We therefore opted to investigate further three siRNA pools that reduced the ability of p53 to activate expression of both PG13 and Bax reporters, and two siRNA pools that selectively inhibited the expression of the PG13 reporter. These siRNAs targeted genes for USP24, USP 36 and USP 42 (reduce activation of PG13 and Bax), and USP15 and USP16 (reduce activation of PG13 only).

To begin to validate the effect of the siRNA pools, we tested the four oligonucleotides in each pool individually for their ability to modulate the p53 response. The most consistent results were obtained using siRNA to knock down the expression of USP15 and USP42 (Figure 1), although reduced activation of the Bax promoter was less clear. Validation of these effects in other reporter cell lines and validation of the extent of knock down of USP15 and USP42 expression is presently underway.

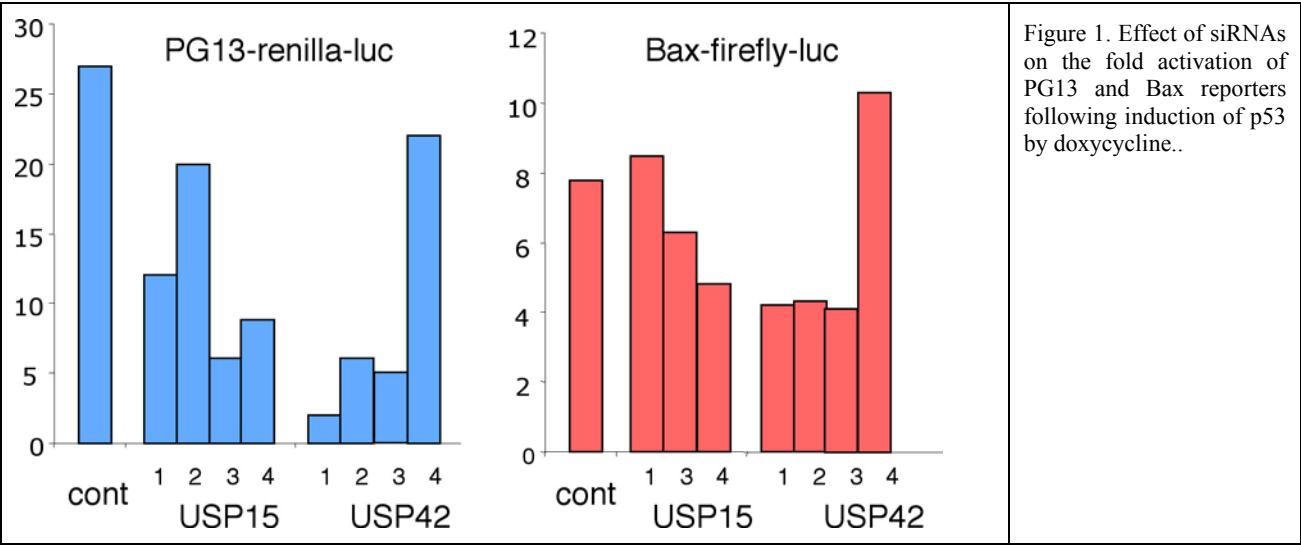


Figure 1. Effect of siRNAs on the fold activation of PG13 and Bax reporters following induction of p53 by doxycycline..

These initial results suggested that knock down of both USP15 and USP42 could affect p53 activity. We therefore have begun to investigate what the basis of this reduced activity might be. Preliminary studies looking at p53 expression levels following doxycycline induction in Saos-2 cells suggest that knock down of USP42 reduces the amount of p53 protein – possibly by reducing the stability of p53 – while knock down of USP15 did not clearly affect p53 protein levels (Figure 2). However, the activation of expression of the p53 target gene Mdm2 was reduced by both USP15 and USP42 knock down.

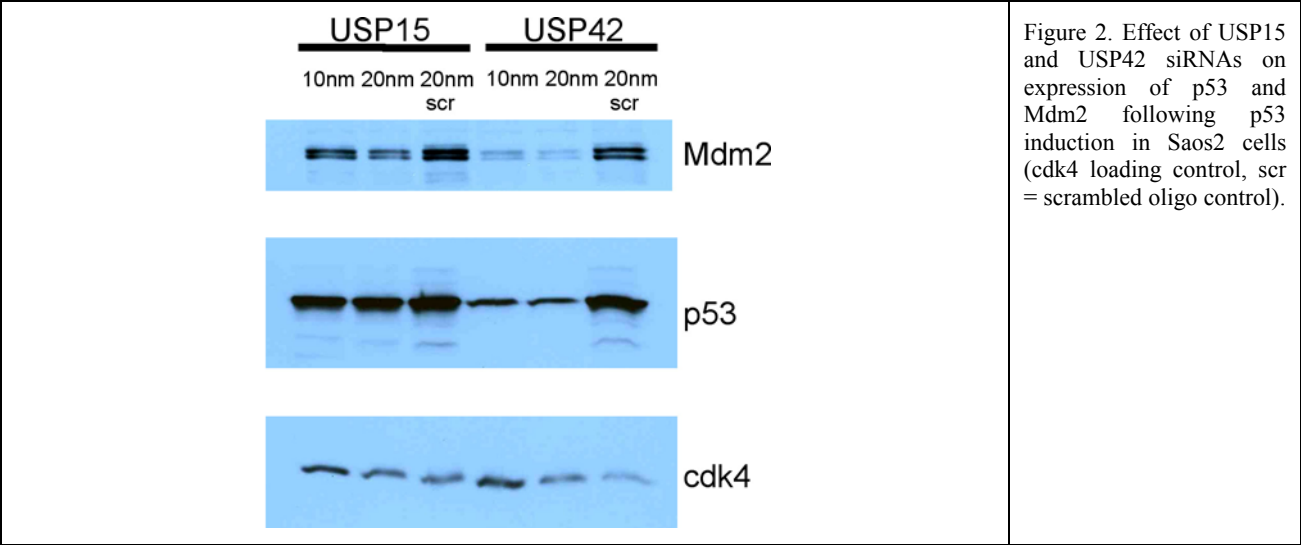


Figure 2. Effect of USP15 and USP42 siRNAs on expression of p53 and Mdm2 following p53 induction in Saos2 cells (cdk4 loading control, scr = scrambled oligo control).

As mentioned above, we have also developed a screening system based on transient transfection of U2OS cells using a pIG3-firefly-luciferase reporter. Screening of the entire human 8k-shRNA library on a one-by-one basis yielded 3 known p53 inhibitors (Mdm2, SIRT1, Twist1) and 4 novel putative inhibitors of p53, namely the gene products of CRMP2, RPS6KA4, RUNDC1 and GAS41. We are in the process of functionally validating these 4 candidate genes. Transient expression of CRMP2, RPS6KA4, RUNDC1 and, to a lesser extent also GAS41, were found to inhibit p53 luciferase reporter constructs for the p53 targets p21, PUMA, Noxa, and Mdm2 (Figure 3A). While overexpression of CRMP2, RPS6KA4, RUNDC1 had no effect cellular p53 levels, GAS41 reduced p53 levels in a manner comparable to Mdm2 (Figure 3B). Importantly, stable overexpression of any of these 4 candidate genes significantly reduced cisplatin-induced apoptosis in U2OS cells (Figure 3C). Together, these findings establish CRMP2, RPS6KA4, RUNDC1 and GAS41 as novel antagonists of p53 function. Ongoing work is aimed at further understanding the role of these genes in p53 regulation and cancer.

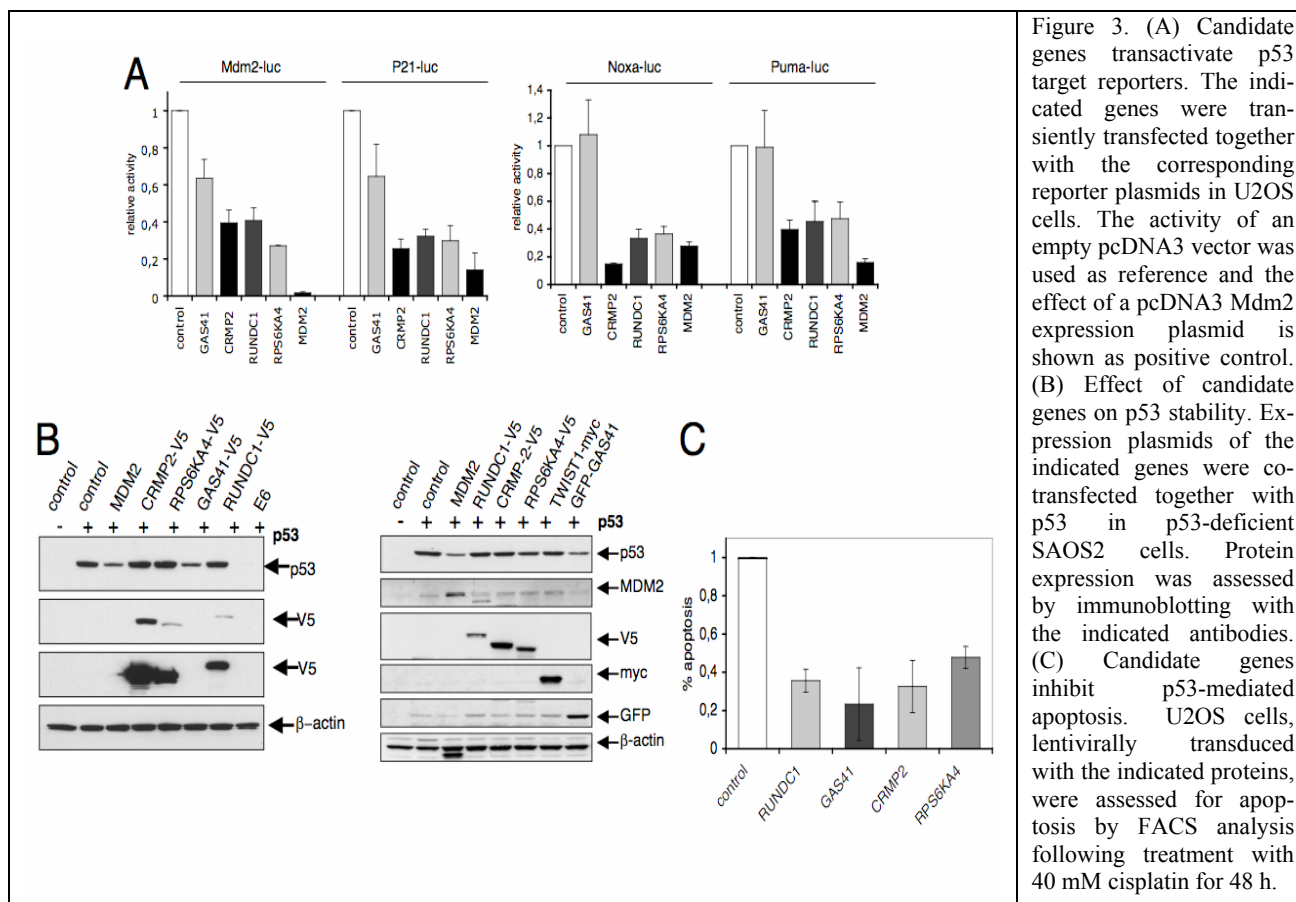


Figure 3. (A) Candidate genes transactivate p53 target reporters. The indicated genes were transiently transfected together with the corresponding reporter plasmids in U2OS cells. The activity of an empty pcDNA3 vector was used as reference and the effect of a pcDNA3 Mdm2 expression plasmid is shown as positive control. (B) Effect of candidate genes on p53 stability. Expression plasmids of the indicated genes were co-transfected together with p53 in p53-deficient SAOS2 cells. Protein expression was assessed by immunoblotting with the indicated antibodies. (C) Candidate genes inhibit p53-mediated apoptosis. U2OS cells, lentivirally transduced with the indicated proteins, were assessed for apoptosis by FACS analysis following treatment with 40 mM cisplatin for 48 h.

Objectives

- Verify system suitable screening PG13 and Bax promoter activity in the same cell
- Identify novel regulators of the p53 pathway using RNAi libraries and p53 reporters
- Characterisation of the respective gene products

Third Annual Periodic Activity Report (2006)

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 10.1	Generation of human epithelial cells that carry tet-repressor	Achieved	M 10.1	12
	T 10.1.2	Generation of GFP/RFP reporter constructs for p53 target genes p21 and Bax	Achieved	M 10.1	12
	T 10.1.3	Generation of human epithelial cells that carry for p53 target genes p21 and Bax	Achieved	M 10.1	12
	T 10.1.4	Generation of human epithelial cells that carry for p53 target genes p21 and Bax in addition to the tet-repressor	Achieved	M 10.1	12
	T 10.2	Testing cell lines to assess suitability for use in screens	Achieved	M 10.2	15
	T 10.3	Screening of cell lines expressing p53 target genes using the high-titer RNAi libraries	Not started yet	M 10.3	21
	T 10.3.2	Screening of cell lines expressing p53 target genes using the ERMtag retroviral vector	Cancelled	M 10.4	21
	T 10.4	Initial characterisation of gene products identified in T 10.3	Not started yet	M 10.5	24
	T 10.5	Validation of the identified genes in regulation of p53-target gene expression in tissue culture systems	Not started yet	M 10.6	24
Update 12-30	T 10.6	Establishing of p21-renilla Bax-firefly luciferase system	Achieved	M 10.7	18
	T 10.7	Establishing of PG13-renilla Bax-firefly luciferase system	Achieved	M 10.8	33
	T 10.7.2	Generation of cell lines stably expressing renilla and firefly luciferase reporters	Not started yet	M 10.8.2	30
	T 10.8	Verification of PG13-renilla Bax-firefly luciferase system for suitability for screening	In progress	M 10.8	33
Update 25-42	T 10.7.2	Generation of cell lines stably expressing renilla and firefly luciferase reporters	Achieved	M 10.8.2	30
	T 10.8	Verification of PG13-renilla Bax-firefly luciferase system for suitability for screening	Achieved	M 10.8	33
	T 10.3	Screening of cell lines expressing p53 target genes using the high titre RNAi libraries	Not started yet	M 10.3	42
	T 10.3.1	Screening of cell lines expressing p53 target genes using siRNA oligonucleotide libraries	Achieved	M 10.3	36
	T 10.4	Characterisation of genes identified in T10.3.1	In progress	M 10.5	48
	T 10.5	Validation of the identified genes in regulation of p53-target gene expression in tissue culture systems	In progress	M 10.6	48

Cluster 2: WP 11

Identification of novel regulators of PUMA expression (BICR)

The main objective of WP 11 is to identify novel regulators of PUMA expression by screening cell lines stably expressing a GFP reporter under the control of the PUMA promoter. As previously reported, we generated and validated a suitable RPE reporter cell line, and were in the process of screening the NKI shRNA library. During 2006 we have focused on carrying out the screens with the shRNA library and trying to isolate and identify some genes whose down-regulation would alter PUMA expression.

We have established a human epithelial cell line stably expressing the PUMA-GFP reporter as well as the murine ecotropic receptor. We have shown that infecting these cells with virus expressing shRNA against p53 successfully inhibited the Adriamycin induced upregulation of p53 activity. This consistently resulted in 40-50% of the treated cells exhibiting a reduced GFP expression level (as a read-out of activation of PUMA-promoter)(pRSp53) compared to the control (pRS) 1 (6-7%) (Figure 1). Repeating the same experiment with test pools (containing p53 shRNA) or another pool spiked with p53 shRNA, resulted in 11-14% of cells (eg Pool 1) with reduced GFP expression compared to control (pRS) or non-responsive pools (Pool 5) (Figure 1).

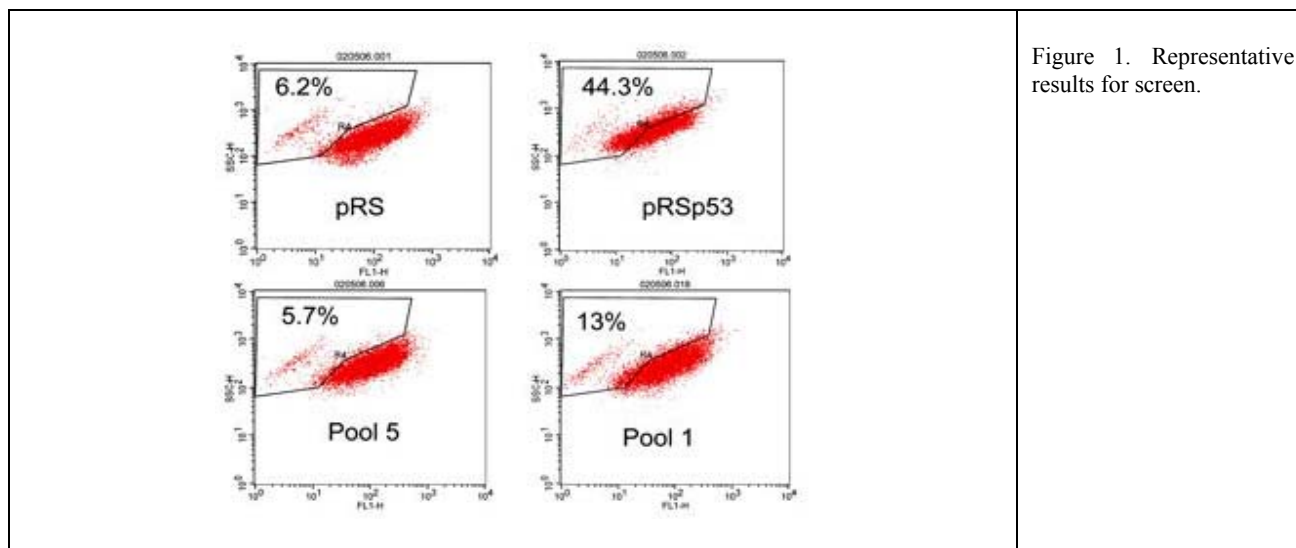


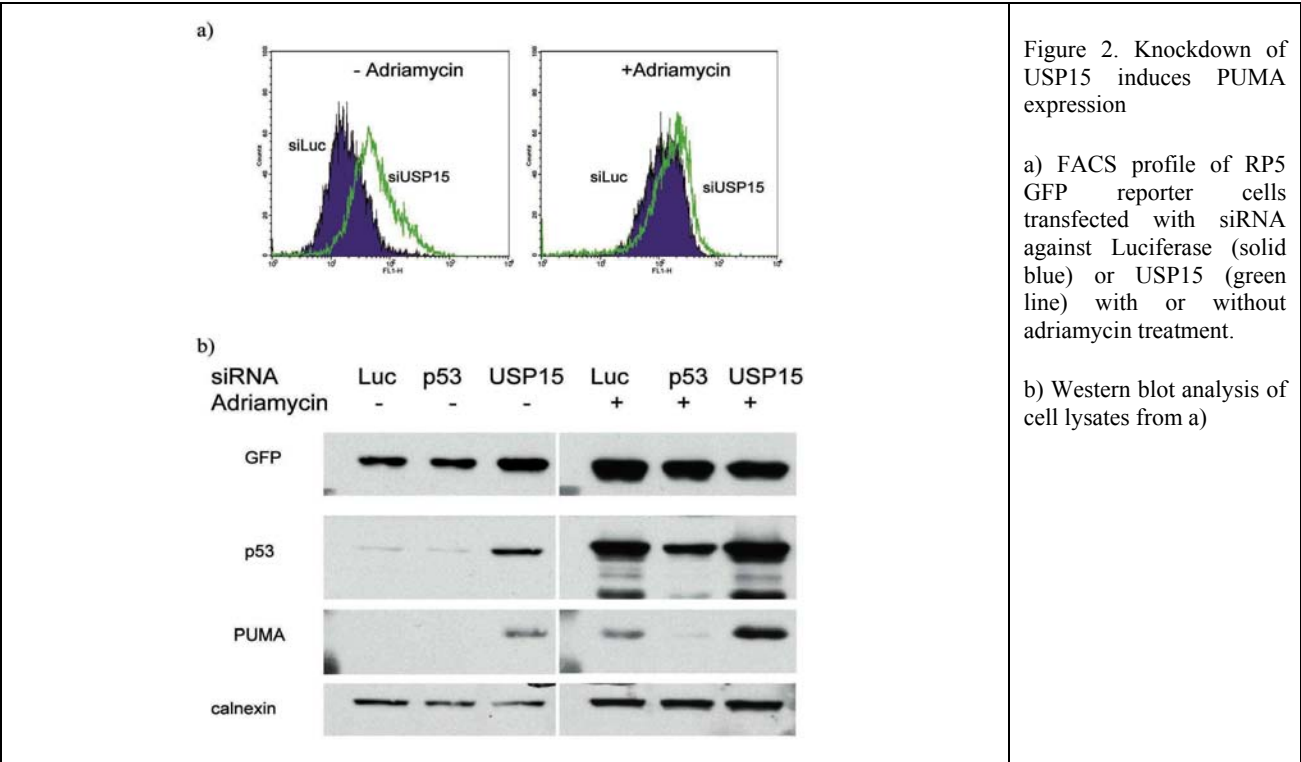
Figure 1. Representative results for screen.

Thus, we established a system whereby we are able to identify pools that contain potential shRNA sequences that have an effect on Adriamycin induced PUMA-promoter activation. Using this method, we have screened 76 pools of the NKI shRNA library (the remaining 4 pools could not be amplified from the master plate) and identified 3 pools that induce an increase in the population of cells with reduced GFP levels. One of these pools contained an shRNA directed against p53, and so served as a useful internal control for the screen.

Attempts to establish and optimize the conditions that would allow successful amplification and enrichment of the shRNA responsible for the downregulation of PUMA-promoter GFP activity using Pool 1 as a positive control were not successful. However, individual pSUPER constructs representing each of the genes represented in these pools are available from the NKI and we have requested these.

While waiting for access to the individual clones, we have taken a more targeted approach and tested the effects of an siRNA oligonucleotide library directed against DUBs (see WP10). Although we were unable to identify any siRNA oligos that resulted in a decrease in the induction of GFP expression after adriamycin treatment, we did identify one siRNA, targeting USP15, that led to an increase in basal GFP expression (Figure 2a). However, GFP expression was only slightly increased after Adriamycin treatment compared to

control siRNA transfected cells (Figure 2a, right). These results were confirmed by Western blot analysis (Figure 2b).



We also examined the effects of USP15 knock down on the activation of endogenous p53 and expression of PUMA (Figure 2b). We found that treatment of these cells with siRNA oligos directed against USP15 led to an increase in endogenous p53 as well as PUMA levels. However, USP15 knock-down did not appear to significantly enhance Adriamycin induced p53 protein levels but did increase endogenous PUMA levels (Fig 2b).

Interestingly, knock down of USP15 was also identified in WP10 as leading to a reduction in the p53-dependent activation of expression from the PG13 promoter, but no clear inhibition of expression of the Bax promoter. The results shown here suggest that USP15 KD has a selective effect on p53 promoter activation, but it is also possible that the upregulation of PUMA observed in these cells might be p53-independent.

Objectives

- Create stable PUMA-GFP reporter cell lines in human epithelial cells
- Identify novel regulators of PUMA expression using RNAi libraries

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

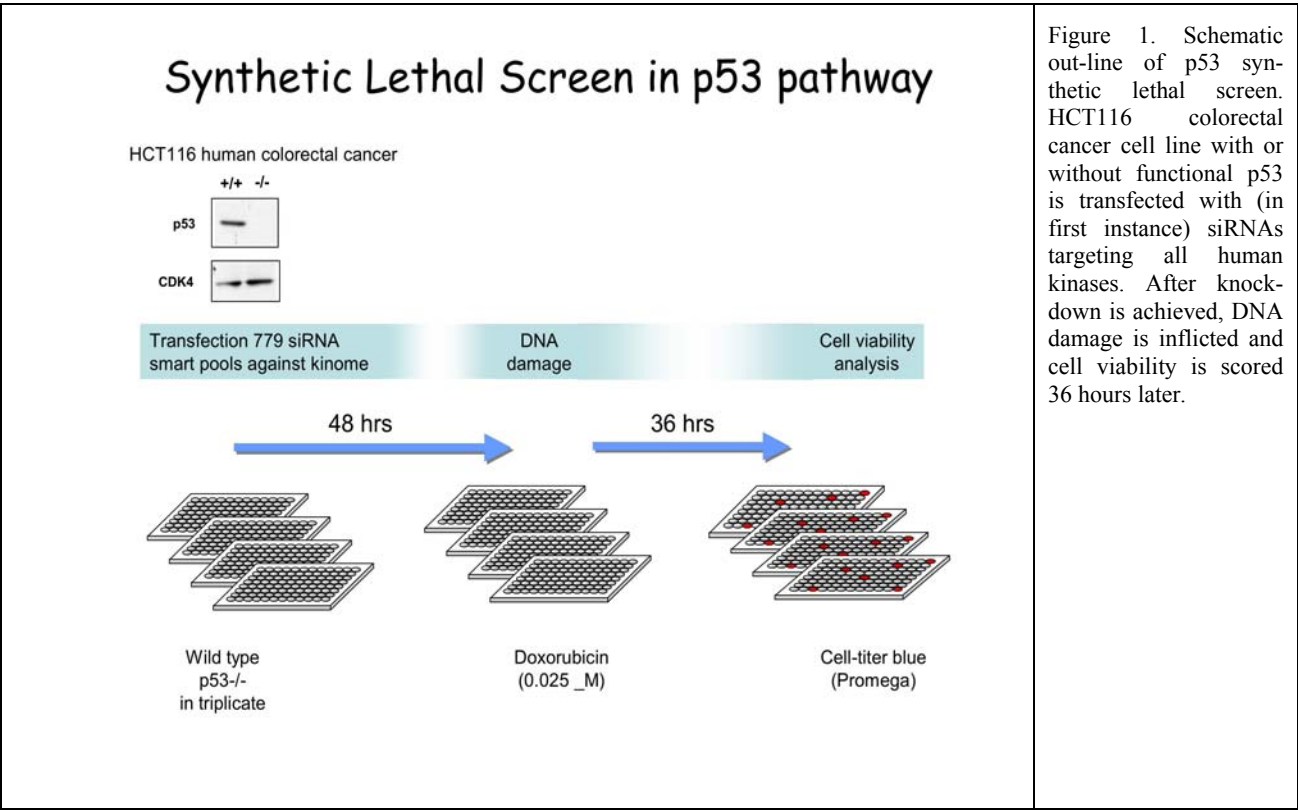
	No.	Task name	Status	Associated milestone	Milestone date
Original	T11.1	Generation of PUMA-GFP reporter	Achieved	M 11.1	15
	T11.2	Evaluating responsiveness of reporter	Achieved	M 11.1	15
	T11.3	Generation of stable cell lines expressing PUMA-GFP reporter	Achieved	M 11.1	15
Update 12-30	T11.4	Testing cell lines to assess suitability for use in screens	In progress	M 11.2	18
	T11.5	Screening of cell lines expressing PUMA-GFP reporter using high-titer RNAi libraries	Not started yet	M 11.3	21
	T 11.6	Initial biological characterization of isolated genes	Not started yet	M 11.4	27
	T 11.7	Comparison of identified genes with genes obtained from other workpackages	Not started yet	M 11.5	36
	T 11.8	Analysis of expression of identified genes in primary tumours by database analysis and screening of multiple tissue arrays by ISH.	Not started yet	M 11.6	36
	T 11.9	Validation of the importance of the identified genes in regulation of PUMA expression in tissue culture systems	Not started yet	M 11.7	42
Update 25-42	T11.4	Testing cell lines to assess suitability for use in screens	Achieved	M 11.2	18
	T11.5	Screening of cell lines expressing PUMA-GFP reporter using high-titer RNAi libraries	Achieved	M 11.3	21
	T11.5.1	Identification of shRNAs isolated from RNAi library screen	Not started yet	M 11.3	24
	T11.5.2	Screening of cell line expressing PUMA-GFP reporter using siRNA oligonucleotide library	Achieved	M 11.8	36
	T 11.6	Initial biological characterization of isolated genes (from siRNA oligonucleotide screen)	In progress	M 11.9	48
	T 11.7	Comparison of identified genes with genes obtained from other workpackages	In progress	M 11.9	36
	T 11.8	Analysis of expression of identified genes in primary tumours by database analysis and screening of multiple tissue arrays by ISH.	Not started yet	M 11.6	48
	T 11.9	Validation of the importance of the identified genes in regulation of PUMA expression in tissue culture systems	In progress	M 11.10	48

Cluster 2: WP 12
Identification of synthetic lethal interactions with p53
(NKI2)

The main focus of this WP is to design and perform synthetic lethal screens in matched cell lines with and without p53. Results from these screenings are anticipated to provide novel targets for cancer therapy, in particular for p53-deficient tumours. The original work plan entailed the use of shRNA bar code screens to find synthetic lethal interactions. However, we have revised the strategy in 2005 for this workpackage and decided to use sets of synthetic siRNAs for this project.

Partner NKI-2 has obtained isogenic derivatives from the human colon carcinoma cell line HCT116 in which the p53 gene is deleted by homologous recombination (Bunz et al., Science 282, p 1497, 1998). In the 2005 progress report, we described that we started on an alternative strategy for the synthetic lethal screens: transfection of siRNAs into 96-well plates. For this purpose, we have obtained a set of 21,000 Dharmacon “smart pools”, each having 4 siRNAs against a single gene of interest. In first instance, we tested a set of 779 siRNAs targeting all human kinases. The schematic outline of the screen is shown in Figure 1 and the initial results in Figure 2. As can be seen, some kinases were toxic, irrespective of p53 status (e.g. PLK1). However, knockdown of one candidate, named 6D3, appears hardly toxic in parental HCT116 cells, but is toxic to the p53 null HCT116 cells.

We will follow up on this initial hit with multiple independent siRNAs (to exclude off target effects of RNAi). After this, we will test the candidate in multiple independent cell lines plus or minus p53 to ask if this interaction is strongly dependent on the genetic context of the cell.



Kinase 6D3 siRNA reduces viability specifically in p53^{-/-} cells

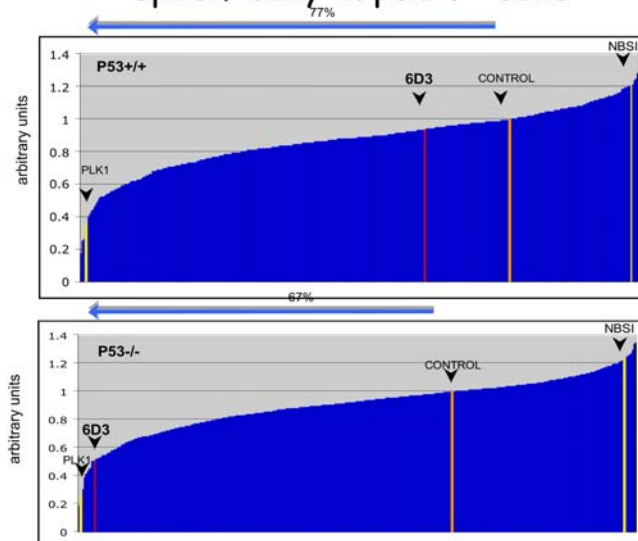


Figure 2. Results of pilot synthetic lethal screen with p53 in HCT116 cells with or without p53. Plotted is cell viability after transfection of siRNAs for 779 human kinases in wild type and p53 null HCT 116 cells. Kinase 6D3 shows little toxicity in parental HCT116, but appears toxic to p53 null HCT 116.

Objectives (revised due to change of strategy)

- Identification of synthetic lethal interactions with p53
- Validation of identified synthetic lethal interactions
- Study of molecular basis for synthetic lethality

Third Annual Periodic Activity Report (2006)

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

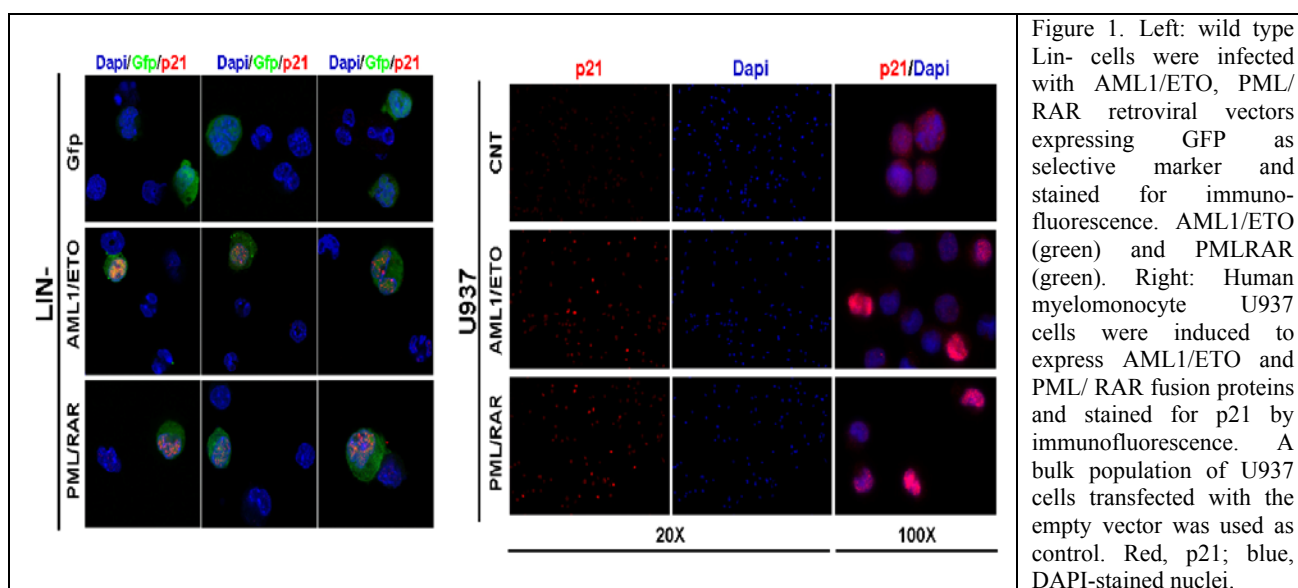
	No.	Task name	Status	Associated milestone	Milestone date
Original	T 12.1a	Generation of human MCF-7 cells with stable knockdown of p53	achieved	M 12.1	12
	T 12.1b	Generation of human U2-OS cells with stable knockdown of p53	achieved	M 12.1	12
	T 12.2	Perform bar coded RNAi screens to identify synthetic lethal interactions with p53	cancelled, alternative strategy	M 12.2	18
	T 12.3	Perform bar coded RNAi screens to identify synthetic lethal interactions with p53 in the presence of genotoxic stress	cancelled, alternative strategy	M 12.2.	18
	T 12.4	Identify candidate synthetic lethal interactions with p53 Using siRNA sets	achieved	M 12.3	30
Update 12-30	T 12.5	Optimize transfection of siRNA sets in 96-well format	achieved	M 12.2	24
	T 12.6	Establishment of a reliable screening system for p53 synthetic lethal interactions in 96-well plates	achieved	M 12.2	24
	T 12.7	Perform high throughput siRNA screen in p53 +/- and p53-/- cells in 96-well format	achieved	M.12.2	30
	T 12.8	Identify candidate synthetic lethal genes with p53 from siRNA screens	achieved	M 12.3	30
Update 25-42	T 12.9	Validate synthetic lethal hits	in progress	M12.4	42

Cluster 2: WP 13

Identification of activators of the p53 pathway in cells expressing haematopoietic oncogenic kinases (IEO2)

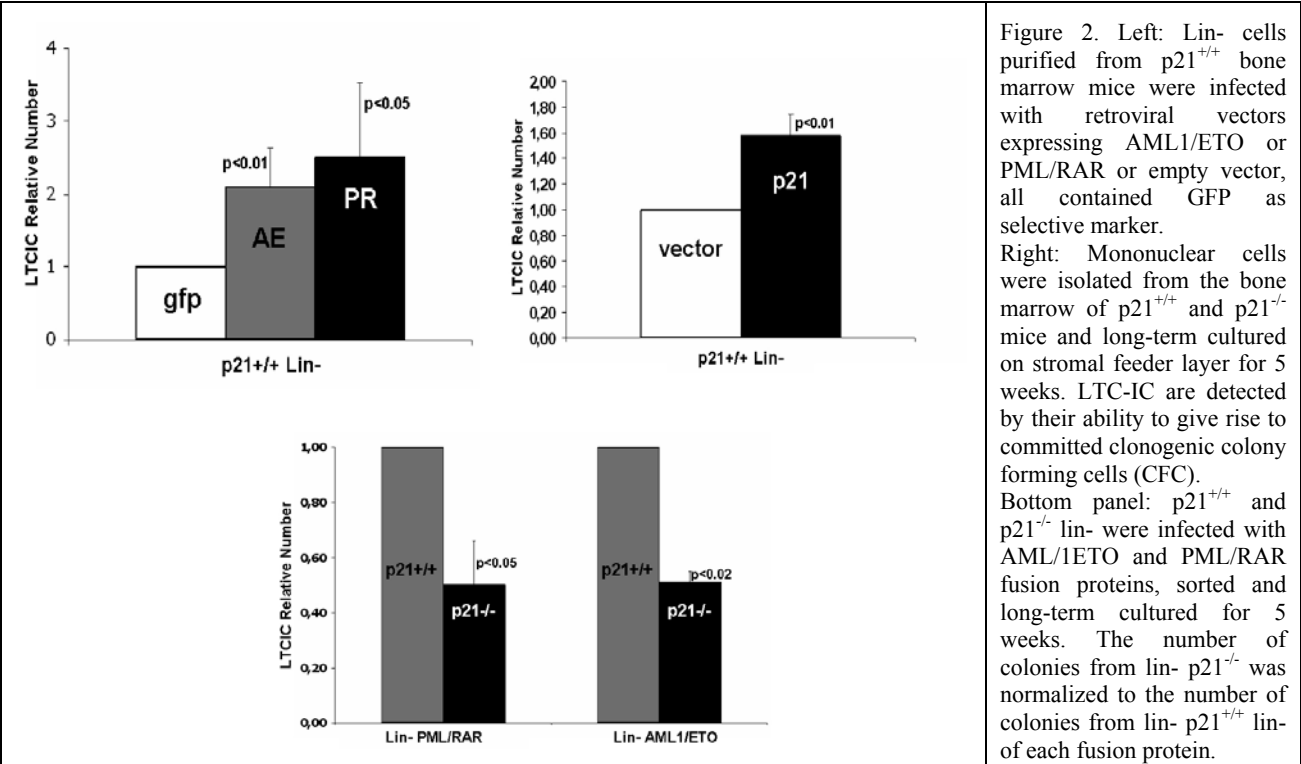
p53 mutations are infrequently found in haematopoietic tumours, suggesting that additional mechanisms of p53 inactivation might be involved. Within this work package, we plan to investigate the molecular pathways underlying the p53-inhibitory effect exerted by leukaemia fusion proteins allowing the identification of pathways critical for cancer development and of new putative target for therapies aimed at reactivating p53 in p53 wild-type tumours. We focused on p53-regulation in haematopoietic stem cells. The data collected during the first two years suggest that quiescent stem cells are pivotal in maintaining the leukaemia population and that expansion of the compartment of quiescent leukaemia stem cells is due to the combined (and opposite) effect of fusion proteins on p53 and p21.

During the last year of activity we confirmed by immunofluorescence studies that leukaemia fusion proteins (AML1/ETO, PML/RAR) induce up-regulation of the p53-target p21 expression both in lin- and U937 cells (Figure 1).

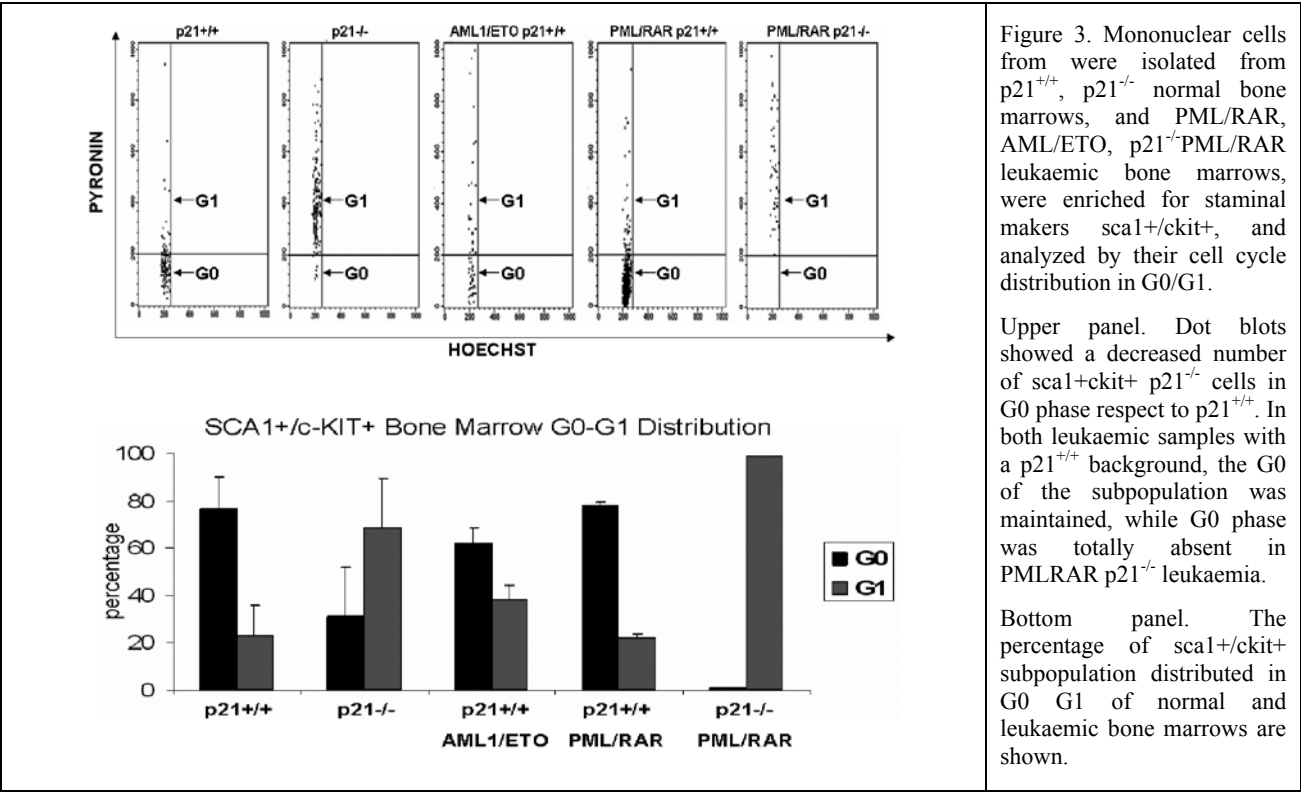


The expression of PML/RAR and AML1/ETO causes, both in MEF and in U937 cells, a cell proliferation block, consistently with the upregulation of a cell cycle inhibitor like p21. Interestingly ectopic expression of the fusion proteins in WT haematopoietic stem cells induces the expansion of the stem cell compartment, as evaluated by the long-term culture initiating-cell (LTC-IC) assay, exactly as ectopic expression of p21. Moreover, the expansion of the stem cell compartment appreciated after the expression of the chimeras, is no more evident in a p21^{-/-} background (Figure 2).

Additionally we investigated the cell cycle profiles of both wt and p21 haematopoietic stem cells in normal or leukaemic mice.



As shown in Figure 3, p21^{-/-} stem cells from normal mice are almost deficient in quiescent cells respect to their p21^{+/+} counterpart.



Third Annual Periodic Activity Report (2006)

The cell cycle distribution that can be appreciated in AML1/ETO and PML/RAR dependent leukaemias in a p21^{+/+} background resembles the normal distribution but, interestingly, in a p21^{-/-} background we can appreciate that PML/RAR leukaemias encounters a rapid exhaustion of the G0 staminal compartment with a simultaneous increase in the cycling compartment, reinforcing the previously experiments that showed that p21^{-/-} PML/RAR leukaemias are not able to transplant. These experiments again support our hypothesis that p21 has a paramount role for the maintenance of the staminal compartment.

Objectives

- Identification of pathways critical for cancer development
- New putative target for therapies aimed at reactivating p53 in p53 wild-type tumours

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 13.1	Generation of human U2OS osteosarcoma cell line expressing a reporter gene (GFP) under the control of a p53 dependent promoter (pGL13): U2OS-GFP.	Achieved	M 13.1	9
	T 13.2	Generation of U2OS-GFP cell lines with inducible expression of NPM-ALK or MDM4.	Achieved-Not useful	M 13.2	15
	T 13.3	Screening of genes whose down regulation reactivate p53, using the above-described cell lines infected with high titers RNAi libraries.	Cancelled, alternative strategy	M 13.3	36
	T 13.4	Generation of a U2OS-GFP cell line stably expressing the tet-repressor.	Achieved	M 13.1	9
	T 13.5	Generation of U2OS-GFP+tet-repressor cell lines with inducible expression of NPM-ALK or MDM4	Achieved-Not useful	M 13.2	15
	T 13.6	Screening of genes that reactivate p53 activity using the cell lines described in task 14.5, using the ERMTAG system.	Cancelled, alternative strategy	M 13.3	36
Update 12-30	T 13.7	Identification and validation of relevant genes coming from the screenings described in task 1 and 2	Cancelled, alternative strategy	M 13.4	30
	T 13.8	Setting up of useful biological systems to be used both to study the pathways involving p53, the selected genes and NPM/ALK, and to test the possibility of using these new genes as therapeutic targets	Cancelled, alternative strategy	M 13.5	48
	T 13.9	Identification of leukaemic stem cells (LSC)	Achieved	M 13.4	36
	T 13.10	Role of LSC in the maintenance of the leukaemic clone	Achieved/ In progress	M 13.4	48
Update 25-42	T 13.11	Analysis of cell cycle properties of leukaemic cells in vivo	Achieved	M 13.4	36
	T 13.12	Function of quiescent LSCs	Achieved/In progress	M13.4	48
	T 13.13	Set-up optimal culture conditions for the retrovirus-mediated infection of leukaemia cells in LTC-IC assays	Achieved/In progress	M 13.4	40
	T13.14	Infection of p21 ^{-/-} leukaemia cells with the bar-code library to select revertants	Cancelled, alternative strategy	M 13.4	36
	T 13.15	<i>p21^{-/-} leukaemic cells transduced with the library injection into mice to further select revertant with transplantation potential.</i>	Cancelled, alternative strategy	M 13.5	48

SUMMARY of research activities during 2006
Cluster 3 (WPs 14-17): The pRB Pathway (NKI3, UKBH, CNIO1, IEO2
(coordinator))

Genetic and biochemical data have placed pRB in a linear pathway, which is deregulated in most human cancers (Hanahan and Weinberg, 2000). This pathway includes cyclin dependent kinase (CDK) inhibitors of the INK4 family, which act as negative regulators of cell proliferation, and the positively acting D-type cyclins that form active kinase complexes in association with CDK4 or CDK6. Phosphorylation of pRB by the CDKs abolishes its activity. The activity of the CDKs, and therefore of pRB, is regulated by external mitogenic and anti-mitogenic factors. It is currently believed that inactivation of the pRB pathway is an obligatory step in cancer that renders cells insensitive to anti-mitogenic signals (Hanahan and Weinberg, 2000). The biochemical mechanism by which pRB is restricting cell proliferation is widely believed to involve protein-protein interactions. Several hundred proteins have been reported to bind to pRB, however, the relevance of most of these interactions is poorly understood. The most studied and the best understood targets for pRB are members of the E2F transcription factor family. Due to the deregulation of the pRB pathway in human tumours, several pharmaceutical and biotechnology companies have initiated programmes to target the specific components in the pathway by developing inhibitors to CDK4 or CDK6 - the only enzymatic activities in the pathway. These inhibitors are still in the developmental phase. Moreover, gene therapy approaches using vectors containing pRB or p16 are under development as well as inhibitors of E2F activity. None of these therapeutics have entered human clinical trials and it is at present unknown if these approaches will be successful. Thus, the main aims of this activity is to identify novel regulators of the pRB pathway using either the p16INK4A promoter (WP 14) or an E2F regulated promoter as functional readouts (WP 15) and to perform the experiments required to understand if they can represent novel targets for therapeutic intervention.

During 2006 the activity of WP14 focused on optimizing the conditions for the use of the ERM viruses to screen for regulators of the Sonic Hedgehog pathway (8xGli-GFP) since p16Ink4a locus reporters did not give a good signal-to-noise ratio. These viruses contain a cassette that has several features that should improve the standard retroviral insertion mutagenesis screen. The cloning of genes that were targeted by the ERM viruses was started, in order to validate which ones are capable of activating the Gli-reporter and thus Shh signalling. In addition, since no predicted candidates have been identified so far; more exhaustive sequencing is currently carried out.

During the early stages of 2006 WP 15 developed a system to automate high throughput screening processes based on fluorescence microscopy in single well format in 96 well microtitre plates (mtps) with the aim of identifying novel regulators of the E2F/Rb pathway using an arrayed version of the full NKI shRNA plasmid library. To this end a stable cell line containing GFP controlled by a minimal CCNA2 promoter has been generated and has been adapted to the HTS system. A number of constructs capable of altering the expression of the GFP reporter was observed.

WP 16 is based on the recent very exciting finding by one of our partners that pRB and its family members p107 and p130 have a role in maintaining telomeres in the absence of telomerase activity (also called ALT). A variety of techniques, including mouse models and RNAi libraries is being used in this project to understand the mechanisms of ALT, the interplay with the pRB pathway and potential novel therapeutic targets.

Several shRNA inserts which a) upon transfection of the HeLa reporter cell line that carries a luciferase reporter whose activity is epigenetically suppressed due to spreading of heterochromatin from an adjacent telomeric repeat domain, enhanced luciferase activity, at the same time, b) induced a detectable loss of telomeric heterochromatin as evidenced by chromatin immunoprecipitation (ChIP) analysis of HeLa cell lysates showing an increase of triacetylation of lysine 9 at histone 3 (Ac3K9H3) and a concomitant decrease

Third Annual Periodic Activity Report (2006)

of trimethylation of lysine 20 at histone 4 (Me3K20H4) at the telomere have been isolated. In addition, relevance of the siRNA pathway for telomere function has been demonstrated.

In the final WP (WP 17) of this cluster, the objective was to characterize genetically the check-point response induced by expression of the NPM-ALK oncogene in normal cells. Lately, it was decided to concentrate the attention on the signalling potential of the NPM component of NPM-ALK.

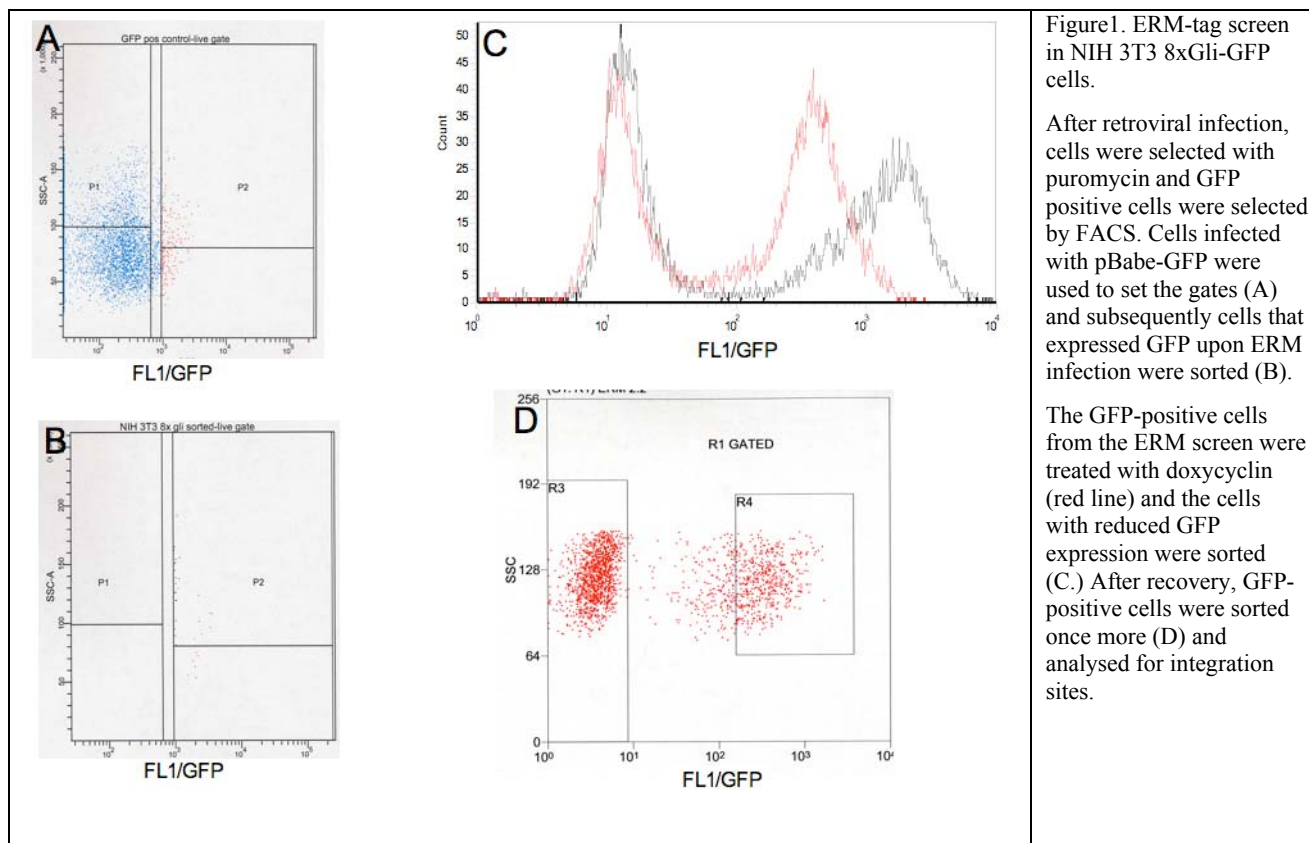
During 2006 the role of NPM wild type in normal cells and the pathological activity of the AML associated NPM mutant was addressed. It was demonstrated that NPM mutants isolated in myeloid leukemias are characterized by an altered cytoplasmic localization dictated by gain-of-function mutations that create a functional Nuclear Export Signal. The direct consequence of this mislocalization is an altered cytoplasmic distribution of the protein Arf. This abnormal Arf localization is not physiological and causes an increased instability for the protein, which is then unable to perform its main function: to activate p53 in response to oncogenic stress, which is believed to be one of the main events in tumor development. This suggests that Arf cytoplasmic sequestration by NPM mutants could represent a paramount event in the neoplastic transformation process. This hypothesis has been confirmed by the expression of oncogenes like Ras and Myc in cells lacking NPM: NPM^{-/-} MEFs are indeed more susceptible to transformation by c-myc.

Cluster 3: WP14

Development of ERMtag screens to identify pathway-specific cancer genes in mammalian cells (NKI3)

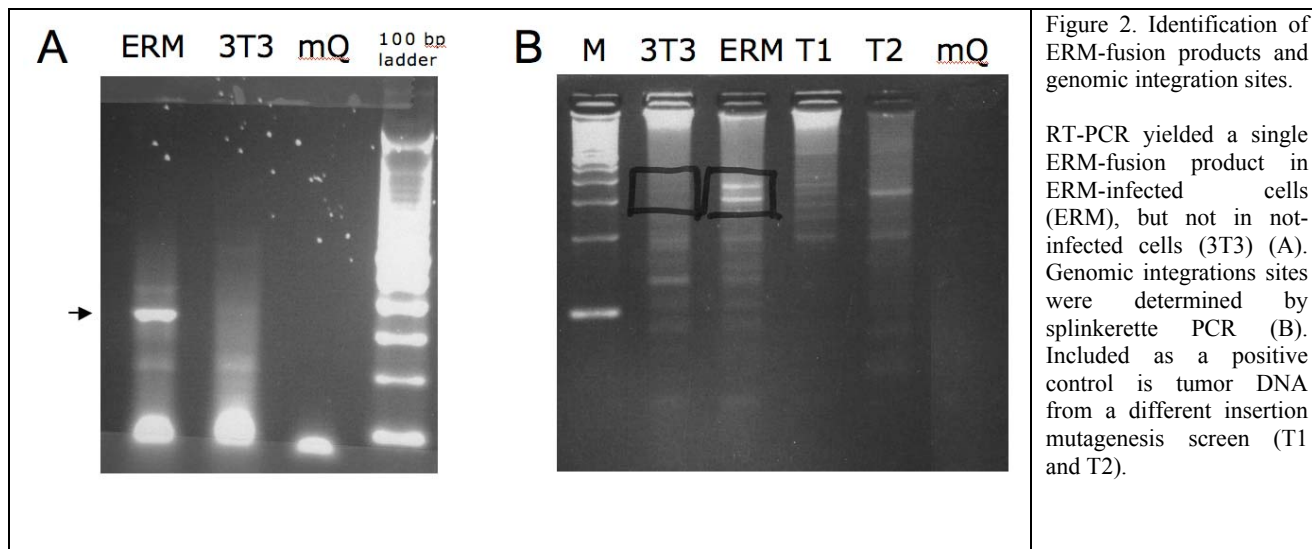
As was reported previously, we have tested several reporters to screen for regulators of the p16Ink4a locus. Since these reporters did not give a good signal-to-noise ratio, we have switched to a reporter for the Sonic Hedgehog pathway (8xGli-GFP). After stable integration of this reporter into NIH 3T3 cells, we subjected these cells to an Enhanced Retroviral Mutagenesis (ERM) screen.

During 2006 we have focused on optimizing the conditions for the use of the ERM viruses. These viruses contain a cassette that has several features that should improve the standard retroviral insertion mutagenesis screen. First, it contains a doxycyclin-responsive promoter, that allows the inhibition retroviral promoter activity, so that false-positive cells can be discarded. Furthermore, this cassette contains a splice-donor site, facilitating the occurrence of fusion transcripts, which can be more readily identified than genomic integrations. In addition, the cassette contains an AU1-epitope-tag to quickly screen for fusion products by Western Blot.



In this initial screen, we have infected 3T3 8xGli-GFP cells with three ERM viruses, with the cassette in all three open reading frames. After infection and puromycin selection, approximately 2 cells/million cells started expressing GFP (figure 1b). We consider this a reasonable efficiency, in view of the fact that random integrations should activate factors that specifically influence the Shh pathway. After a culture period to expand the small population of sorted GFP-positive cells, these cells were subjected to doxycyclin. The ERM

viruses are dependent on VP16 for their activity, which is sequestered by doxycyclin, therefore the cells should lose their GFP expression. Cells that maintain GFP expression in the presence of doxycyclin may have induced GFP expression in a way that is not dependent on the integrated viruses, and are therefore discarded as false positives. In this screen with relatively low complexity, all cells responded to doxycyclin (figure 1c). These cells were sorted again, expanded in the absence of doxycyclin and sorted once more for GFP-positivity (figure 1d). In this case, only a subset of the cells had regained their GFP expression. From these cells, we isolated genomic DNA, RNA and protein.



Antibody staining against the AU-1 epitope on Western Blot did not show any fusion transcripts. However, after RNA isolation and the generation of cDNA with a random primer that included a T7-tag, RT-PCR with an ERM-specific and the T7 primer did show a clear product (figure 2a). Splinkerette PCR on the genomic DNA revealed several predominant bands that have been cloned and sequenced (figure 2b). Currently, we are cloning the genes that were targeted by the ERM viruses, so that we can validate which ones are capable of activating the Gli-reporter and thus Shh signalling. In addition, since no predicted candidates have been identified so far; more exhaustive sequencing is currently carried out. At the same time, we are repeating this screen to obtain a larger pool of cells, since the starting population of the current GFP-positive cell pool was quite small.

In summary, we have demonstrated that an ERM insertion mutagenesis screen is feasible if the signal-to-noise ratio of the reporter is good enough. In addition, we have created protocols for the set-up and analysis of ERM-tag screens, which are available to all members of the consortium. Finally, we are analyzing potential novel regulators of the Shh pathway.

Objectives (revised due to change of strategy)

- Evaluation, optimization and implementation of ERM-tag screens
- Identification and characterization of genes required for induction of the Shh pathway

Third Annual Periodic Activity Report (2006)

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

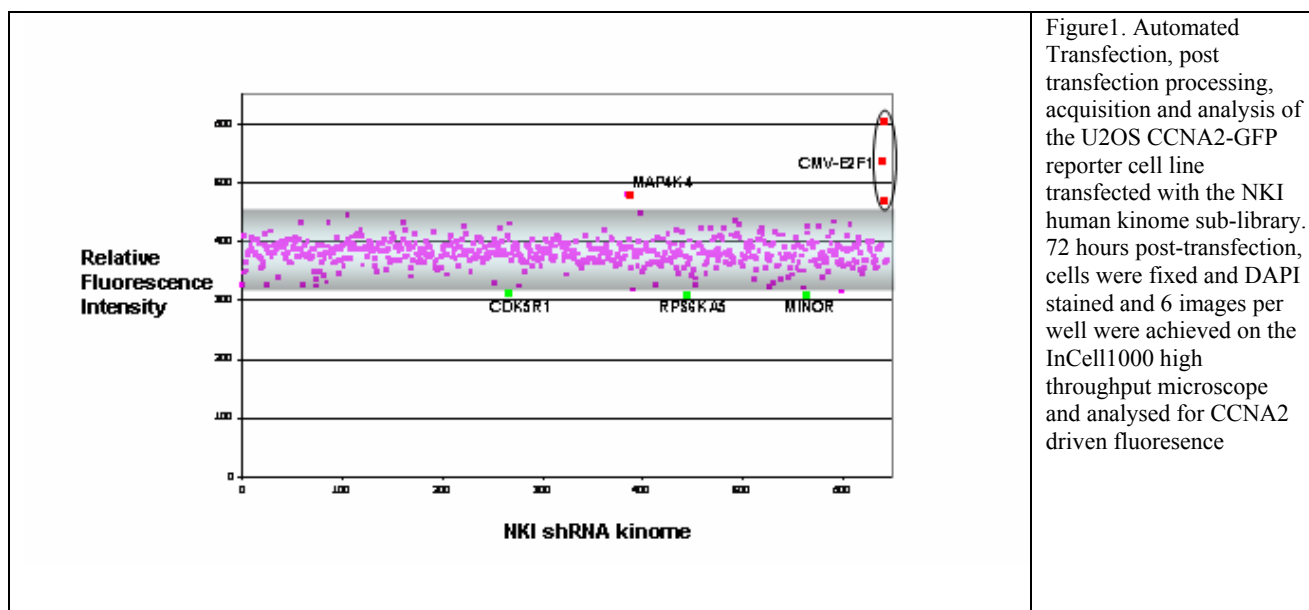
	No.	Task name	Status	Associated milestone	Milestone date
Original	T 14.1	Generation of TERT-immortalized fibroblasts expressing the tet-repressor.	Achieved	M 14.1	6
	T 14.2	Generation of human fibroblast cell lines that carry GFP reporters for p16INK4a in addition to the tet-repressor.	Achieved	M 14.1	6
	T 14.3	Generation of ES cells carrying a targeted replacement of a p16INK4a-GFP reporter into Exon1alpha.	On hold, see text		
	T 14.4	Generation of mouse embryo fibroblasts with the p16INK4a-GFP reporter at the endogenous INK4a locus.	On hold, see text		
	T 14.5	Screening of tet-repressor human cell lines expressing INK4a-GFP using the ERM tag retroviral vector, for positive and negative regulators.	On hold until proven feasible with GLI (see WP3)	M 14.10	30
	T 14.6	Screening of the human p16INK4a-GFP cell line using high-titer RNAi libraries.	In progress	M 14.7	18
	T 14.7	The identified genes will be compared with the genes obtained in the screens in the other workpackages, in particular from WP18 and WP21	Not started yet	M 14.11	30
Update 12-30	T 14.8	The expression of the identified genes in primary tumours will be analysed by database analysis and screening of multiple tumour arrays by ISH.	Not started yet	M 14.11	30
	T 14.9	Testing the feasibility of screening of the human p16INK4a-GFP or luciferase reporters using high-titer RNAi libraries in a pooled format using FACS read-out.	In progress	M 14.7	18
	T 14.10	Screening for positive and negative regulators of p16INK4a using shRNA libraries.	In progress	M 14.7	18
	T 14.11	Recovery and DNA sequencing of shRNAs isolated from the p16INK4a screen.	In progress	M 14.9	30
	T 14.12	Functional validation of the identified genes for their requirement in p16INK4a regulation.	Not started yet	M 14.11	30
Update 25-42	T 14.13	Generation and testing of multimerized p16INK4a-LUC reporter	Achieved	M14.12	36
	T 14.14	Performance of scaled up GLI-GFP reporter ERMtag screen	In progress	M14.13	39
	T 14.15	Performance of INK4a-LUC reporter screen	Not started yet	M14.14	48
	T 14.16	Initial characterization and validation of Gli regulators	In progress	M14.14	48
	T 14.17	Generation of antibodies against validated targets and analysis of their expression in a panel of human brain tumors	Not started yet		48

Cluster 3: WP 15

Targeting E2F using RNAi and ERMtag-based screens (UKBH)

As previously reported, we have been developing a system to automate high throughput screening processes based on fluorescence microscopy in single well format in 96 well microtitre plates (mtps) with the aim of identifying novel regulators of the E2F/Rb pathway using an arrayed version of the full NKI shRNA plasmid library. To this end we have generated a stable cell line containing GFP controlled by a minimal CCNA2 promoter and have been adapting this to a HTS system.

During the early stages of 2006 we installed our automated HT screening system comprising a general liquid handling station and a HT microscope. We have subsequently focused on using the NKI human kinome sub-library in pilot assays to validate our automated screening approach. To this end we have successfully developed a transfection based procedure using Lipofectamine 2000, which is amenable to 96 well mtp assays. The transfection mix is dispensed to cells that were seeded on the system 24 hours prior (not a very clear sentence). We routinely achieve a transfection efficiency of 70% in U2OS cells whilst using this approach (as determined by visualisation of a fluorescent plasmid based marker). The system has been optimised to perform the subsequent post transfection processes including the exchange of transfection media/ fixing of cells/ DAPI staining (which is also easily adaptable to Immunofluorescence based approaches). Following this process plates are delivered automatically into a HT microscope where images are acquired and analysed automatically using a sophisticated image analysis package.



As presented in Figure 1 we have observed a number of constructs altered the expression of the GFP reporter ($\pm 3SD$). As anticipated, CMV driven overexpression of E2F1 led to a detectable increase in CCNA2 driven GFP expression in this cell line. One transfected construct, MAP4K4 (a STE20 like kinase) led to an increase in GFP expression, which was comparable to the overexpressed E2F1 control transfections. MAP4K4 has recently been identified using an RNAi based screen attempting to identify regulators of wound healing as a promigratory kinase, has been shown to be upregulated in tumour lines and is a putative p53 target gene.

Third Annual Periodic Activity Report (2006)

There were also 3 constructs CDK5R1, RPS6KA5 and MINOR that were shown to decrease the expression of the reporter. The characterisation of these genes and a role in E2F regulation is not very well advanced however there is some literature suggesting a role for regulation of cellular proliferation for each of these genes. For example, CDK5R1 (an activator of CDK5) is neuronally expressed and has been proposed to play a role in CDK5 mediated phosphorylation of pRB during neuronal apoptosis (Interestingly, transfected shRNA against CDK5R1 reduced the cell number to below 50% of control transfections). Several early reports have shown that MINOR/NR4A3 (a NUR77 family member) is translocated in a subset of chondrosarcomas and has also been implicated in apoptosis. Furthermore Smooth Muscle Cells derived from NR4A3 null mice display decreased proliferation and diminished CCND1 and CCND2 levels. Lastly RSP6KA5/MSK1 was shown to have a similar effect on GFP expression. MSK1 has been shown to directly repress transcription via phosphorylation of Serine-1 of Histone H2A. Furthermore MSK1 mediates the mitogen- and stress-induced phosphorylation of Histone H3, which displaces HP1 γ from target genes during mitogen activation.

Objectives (revised due to change of strategy)

- Create stable GFP reporter cell lines for human *CCNA2* and/or *EZH2* locus in human cell lines
- Identify novel regulators of the pRB/E2F pathway using RNAi libraries and the E2F reporter gene(s)
- Analysis of positives in cell culture models

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 15.1	Generation of human fibroblasts expressing the tet-repressor	Achieved	M 15.1	6
	T 15.2	Generation of stable cell lines containing a Cyclin A2 or Cyclin E1 GFP reporter	Achieved	M 15.1	6
	T 15.3	Screening of tet-repressor cell lines expressing CCNA2/CCNE1 using the ERM tag retroviral vector	Cancelled, alternative strategy	M 15.2	18
	T 15.4	Screening of CCNE1-GFP, CCNA2-GFP cell lines using high titer RNAi libraries	Awaiting delivery of 2 nd generation arrayed human shRNA library	M 15.3	18
	T 15.5	The identified genes will be compared with the genes obtained in the screens in the other work packages	Not started yet	M 15.4	36
	T 15.6	The expression of the identified genes in primary tumours will be analysed by database analysis and screening of multiple tumour arrays by ISH	Not started yet	M 15.5	42
	T 15.7	Gene copy numbers for selected overexpressed or non-expressed genes will be determined by FISH	Not started yet	M 15.5	42
Update 12-30	T 15.8	Development of a system for automated screening of the human shRNA library	Achieved	M 15.6	18
	T 15.9	Generation of CCNA2 reporter cell lines validated for high throughput screening compatability	Achieved	M 15.7	24
	T 15.10	Generation of human diploid fibroblasts containing EZH2-GFP reporter	Change of strategy Awaiting outcome of CCNA2 screen	M 15.8	30
	T 15.11	Generation of EZH2 GFP reporter cell lines validated for High throughput screening compatability	Change of strategy Awaiting outcome of CCNA2 screen	M 15.9	33
Update 25-42	T 15.12	Establishment and performance of HCS using NKI shRNA human kinome sub-library	Achieved	M 15.10	30
	T 15.13	Characterization of targets identified in kinase sub library screen in cell culture models	In progress	M 15.11	42

Cluster 3: WP 16
Identification of epigenetic regulators of telomere function
CNIO1

The main goal of this WP is to identify and functionally characterize genes that regulate telomere structure and function by epigenetic mechanisms. With this aim in mind, we have performed a genome-wide screen with the NKI shRNA library using a reporter cell line that carries a luciferase reporter whose activity is epigenetically suppressed due to spreading of heterochromatin from an adjacent telomeric repeat domain.

We have isolated several shRNA inserts which a) upon transfection of the HeLa reporter cell line enhanced luciferase activity, at the same time, b) induced a detectable loss of telomeric heterochromatin as evidenced by chromatin immunoprecipitation (ChIP) analysis of HeLa cell lysates showing an increase of triacetylation

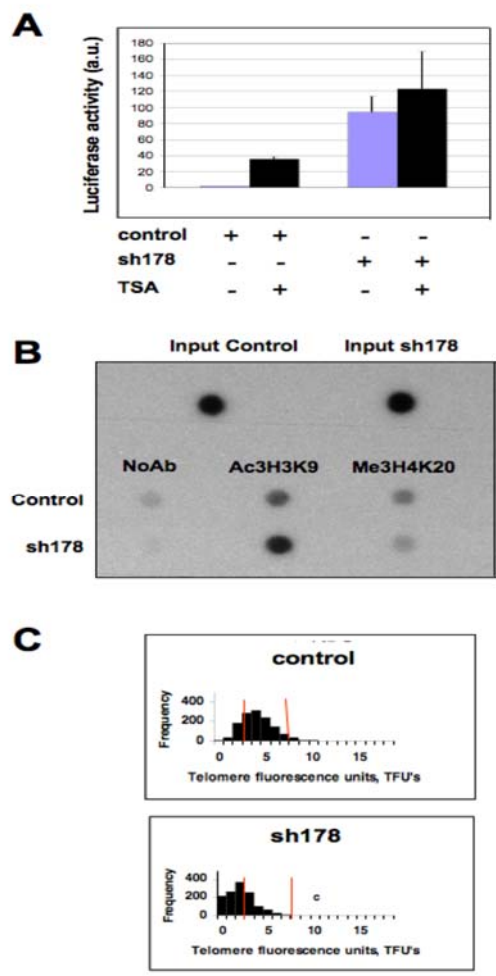


Figure1.

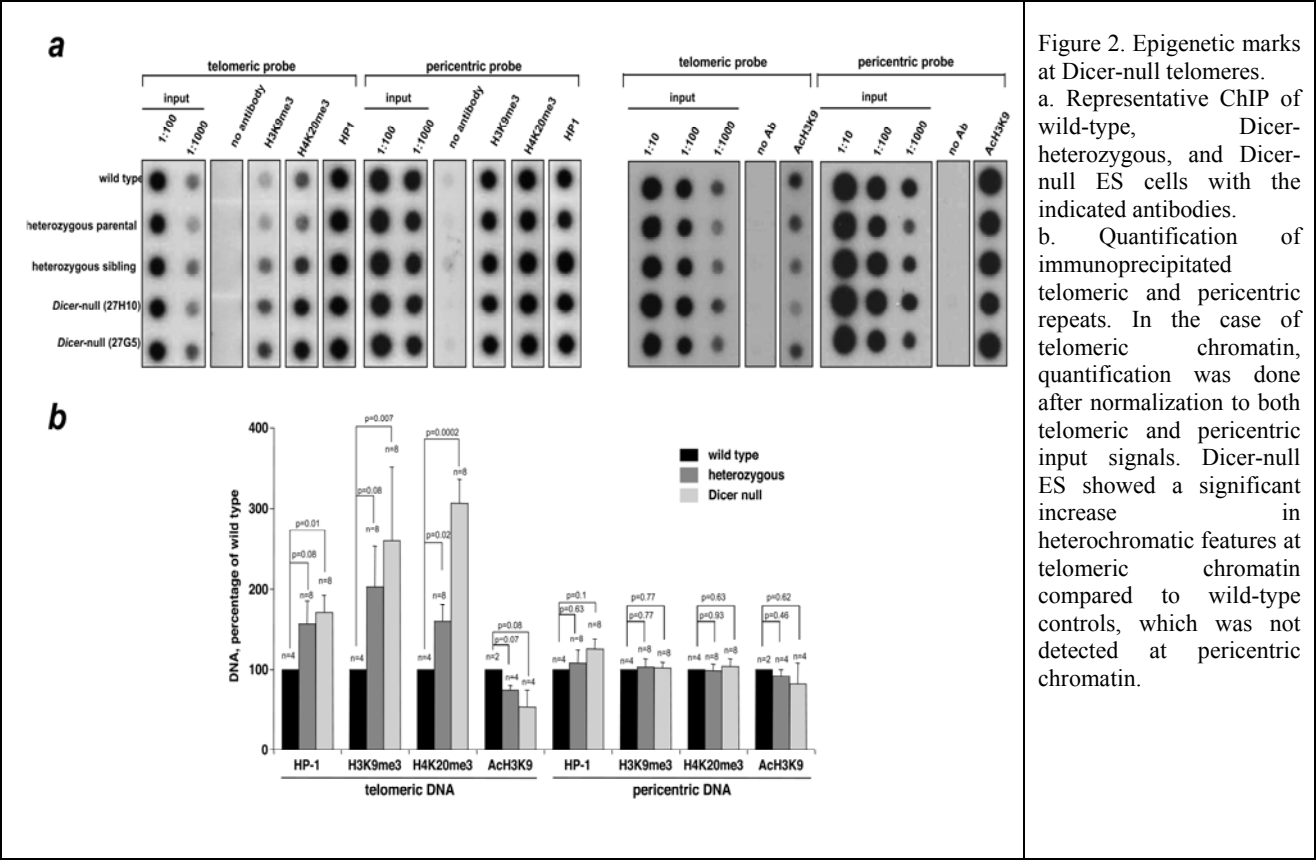
(A) Luciferase activity in lysates of HeLa reporter cells carrying a telomerized luciferase reporter construct and transfected with empty vector (control) or vector containing the shRNA clone 178 (sh178). Cells were cultured for 48 h in the absence (blue columns) or presence (black columns) of the HDAC inhibitor TSA (1 μ M) prior to reporter activity measurements (mean \pm SEM; n=3).

(B) ChIP analysis of HeLa cell lysates transfected with empty vector (control) or vector containing the shRNA clone 178 (sh178). Immunoprecipitation was performed with the indicated antibodies, and telomeric repeats were detected by hybridization with a 32 P-labeled telomeric probe.

(C) Telomere length analysis by quantitative fluorescence in situ hybridization (Q-FISH) of HeLa cell lysates transfected with empty vector (control) or vector containing the shRNA clone 178 (sh178). Mean telomere length for control and sh178 transfected cells were 4.0 and 2.3 kb, respectively.

of lysine 9 at histone 3 (Ac3K9H3) and a concomitant decrease of trimethylation of lysine 20 at histone 4 (Me3K20H4) at the telomere. Validation of these clones yielded one promising candidate (shRNA178), which stimulated the telomerase reporter >100-fold, induced a marked loss of telomeric heterochromatin, and detectable telomere shortening (Figure 1).

In addition, we have recently been able to demonstrate the relevance of the siRNA pathway for telomere function. We found that mouse ES cells deleted for Dicer show aberrantly elongated telomeres compared to wild type controls, concomitant with increased telomeric recombination, which could account for the long-telomere phenotype of these cells. This occurs in the absence of changes in TRF1 and TRF2 expression and with decreased telomerase activity in Dicer-null cells. The long-telomere phenotype of Dicer-null cells is accompanied by an increased density of histone heterochromatic marks and decreased histone acetylation, suggesting that the length of TTAGGG repeats is important to direct heterochromatin assembly at telomeres (Figure 2).



Objective

- Identification of epigenetic regulators of telomere function

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 16.1	Construction of RNAi retroviral vectors to interfere with known activities involved in telomere length maintenance (telomerase, homologous recombination proteins)	Achieved	M16.1	6
	T 16.2	Study the effects on telomere length and function of the different RNAi retroviruses (task 16.1) in cells deficient for the different members of the Rb family of proteins	Achieved	M16.2	12
	T 16.3	Gene array analysis on the different cell systems mentioned above (task 16.2)	Cancelled, alternative strategy	M16.3	18
	T 16.4	Validation of the new targets by “real-time” PCR and RNAi	Cancelled, alternative strategy	M16.4	18
	T 16.5	Screening of the different mouse models and cellular systems using high-titer RNAi libraries for identification of additional targets	Cancelled, alternative strategy	M16.5	18
	T 16.6	Generation of new mouse models	Cancelled, alternative strategy	M16.5	18
Update 12-30	T 16.7	Validation of a screening system aimed at the identification of regulators of telomeric heterochromatin	Achieved	M16.6	15
	T 16.8	Screening for genes that modify telomeric heterochromatin	Achieved	M16.7	21
	T 16.9	Functional validation of the identified genes	In progress	M16.8	30
Update 25-42	T 16.10	Extension of findings on the role of the identified genes as regulators of telomere function in HeLa cells to MEFs	To be started	M16.9	33
	T 16.11	Genetic interaction between the identified genes and other regulators of telomere function such as Rb	To be started	M16.9	36
	T 16.12	Impact of the identified genes on telomere-driven genomic instability, cell growth, immortalization, and transformation	To be started	M16.10	42

Cluster 3: WP 17
Synthetic lethality with Rb loss and p53-independent checkpoints
IEO

As reported previously, the objective of this WP was to characterize genetically the check-point response induced by expression of the NPM-ALK oncogene in normal cells. Lately, we have decided to concentrate our attention on the signalling potential of the NPM component of NPM-ALK, for two reasons: i) NPM-ALK mutants with defective NPM activity induce lymphomas in mice at a markedly longer latency, suggesting that deregulation of NPM contributes to checkpoint regulation and genomic instability; ii) NPMN is frequently mutated in myeloid leukemias, suggesting that NPM has transforming potential in the absence of ALK. We have therefore concentrated our experimental work on the analysis of the physiological function of NPM and biological properties of AML-associated NPM mutants.

During 2006 we have focused on the characterization of the role of NPM wild type in the normal cells and the pathological activity of the AML associated NPM mutant. We recently demonstrated that NPM mutants isolated in myeloid leukemias are characterized by an altered cytoplasmic localization dictated by gain-of-function mutations that create a functional Nuclear Export Signal. The direct consequence of this mislocalization is an altered cytoplasmic distribution of the protein Arf. This abnormal Arf localization is not physiological and causes an increased instability for the protein, which is then unable to perform its main function: to activate p53 in response to oncogenic stress, which is believed to be one of the main events in tumor development. This suggests that Arf cytoplasmic sequestration by NPM mutants could represents a paramount event in the neoplastic trasformation process.

This hypothesis has been confirmed by the expression of oncogenes like Ras and Myc in cells lacking NPM: NPM-/- MEFs are more susceptible to trasformation by c-myc (Figure 1).

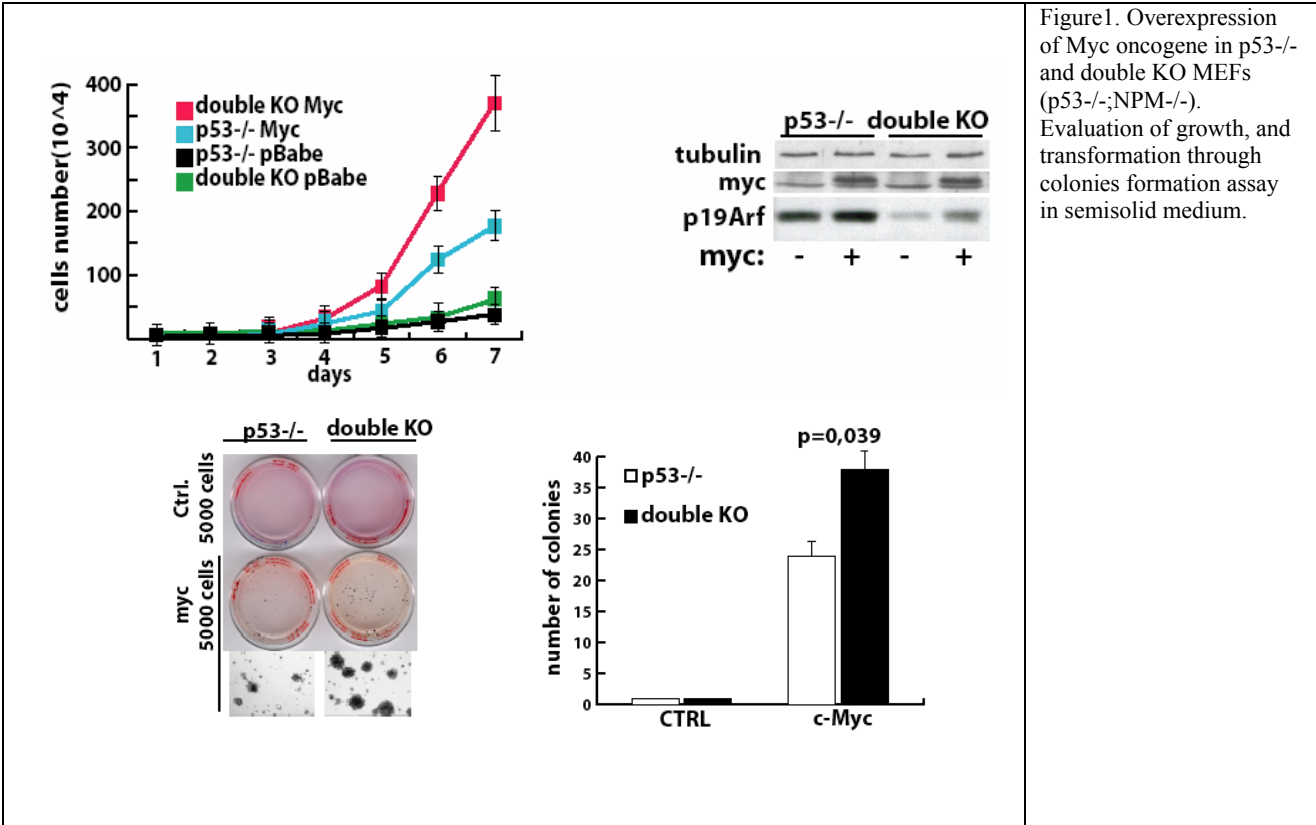
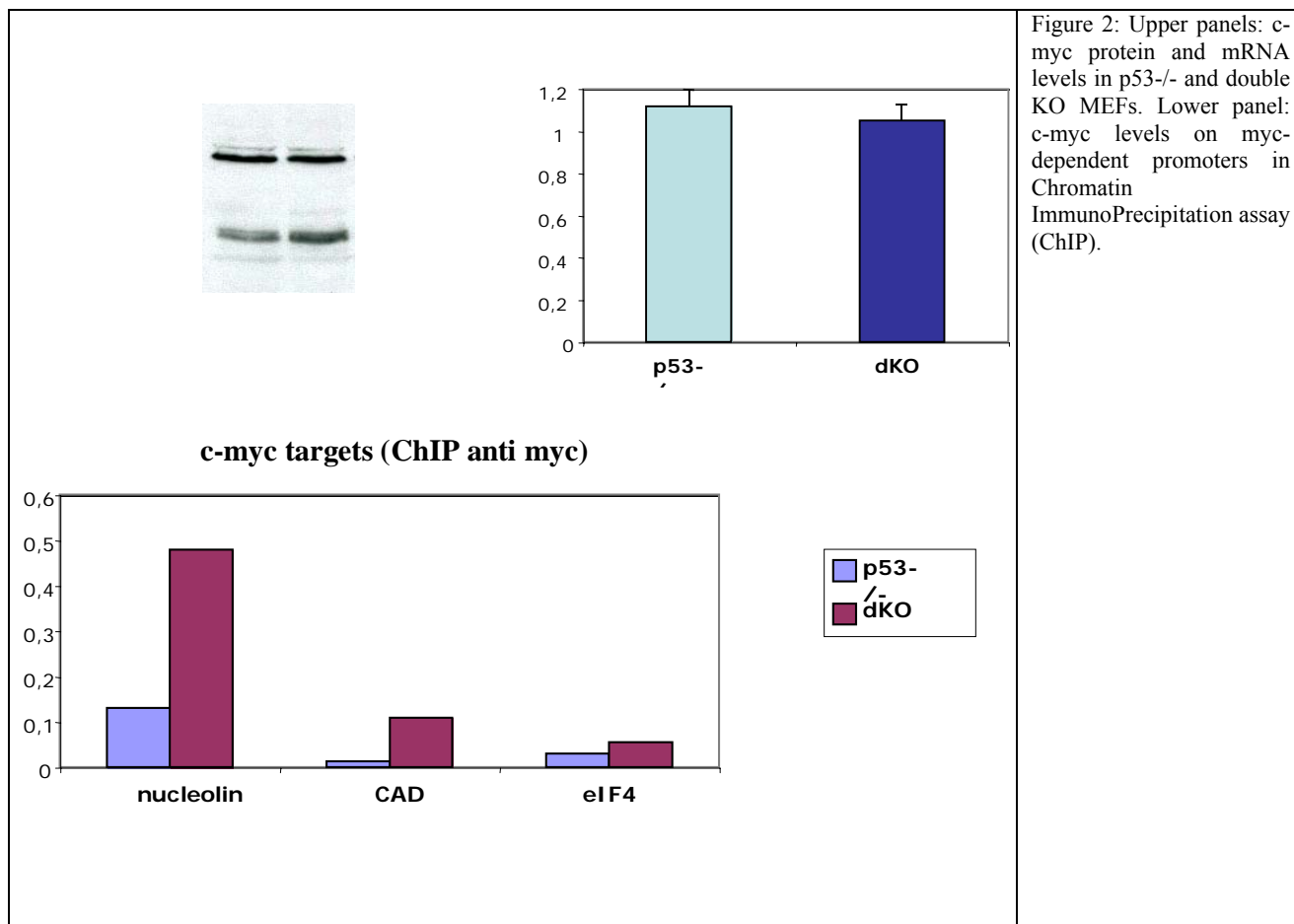


Figure1. Overexpression of Myc oncogene in p53-/- and double KO MEFs (p53-/-;NPM-/-). Evaluation of growth, and transformation through colonies formation assay in semisolid medium.

We decided therefore to investigate in more details the molecular mechanisms at the basis of this increased susceptibility.

Interestingly we found that in the absence of NPM, the stability of c-myc protein is strongly increased (Figure 2), suggesting an additional role for NPM in c-myc degradation. Moreover, we have demonstrated that the absence of NPM is able to increase the recruitment of c-myc on the promoters of its target genes (Figure 2).



Therefore, we are facing a new interesting scenario in which NPM is an important regulator of the cell cycle progression since it can modulate the activity and stability of Arf and also directly the stability and the transcriptional activity of the oncogene c-myc. Importantly, it has been recently demonstrated that oncogene activation causes DNA damage response. Therefore, a possible working hypothesis is that the DNA damage observed in cells lacking NPM is not due to a direct role of NPM in DDR but to an uncontrolled activation of c-myc oncogene.

We know already that the AML-associated NPM mutant interferes with NPM-Arf binding causing Arf delocalization and degradation. It is our intention to deeply investigate the molecular mechanism by which NPM control c-myc activity and in parallel verify if also in this case the NPM mutant interfere with this pathway. If this is the case, the mutant NPM will potentially display important oncogenic activities interfering with both the tumour suppressor Arf and the oncogene c-myc and therefore it becomes an interesting therapeutic target.

Third Annual Periodic Activity Report (2006)

In the next months we will put our efforts in developing a mouse model of NPM-mut dependent Acute Myeloid Leukemia, and in the identification of molecular based strategy of therapeutic intervention specific for human AML bearing NPM mutations.

Objectives

- Development of a mouse model of NPMmut-dependent AML
- Setting-up novel cellular assays for genetic screens using RNAi library approaches
- Characterization of physiological roles of NPM and potential oncogenic activity of AML derived NPM mutants

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 17.1	Generation of human normal fibroblasts (WI38) with impaired Rb function through stable expression of a retroviral construct that express RNAi specific for Rb.	Cancelled, alternative strategy	M 17.1	24
	T 17.2	Infection with high titer RNAi library of Rb-inactive WI38 cells, to identify by negative selection genes whose down regulation display synthetic lethality with Rb loss.	Cancelled, alternative strategy	M 17.2	36
Update 13-30	T 17.3	Generation of primary cells in which cell growth is inhibited by inducible expression of NPM/ALK.	Cancelled, alternative strategy	M 17.1	24
	T 17.4	Infection with high titer RNAi library of NPM/ALK expressing primary cells to identify by positive selection genes whose inactivation allows expression of NPM/ALK.	Cancelled, alternative strategy	M 17.2	36
	T 17.5	Characterization of the NPM knock-out mouse	Achieved	M 17.3	36
	T 17.6	Characterization of the cytoplasmic localization of NPM in myeloid leukemias	Achieved	M 17.4	36
	T 17.7	Analysis of the delocalization and destabilization of the Arf tumor suppressor by the leukemia-associated NPM mutant	Achieved	M 17.4	36
Update 25-42	T 17.8	Generation of a transgenic mouse model in which the expression of the AML associated NPM mutant is under the control of an artificial promoter, and is inducible through a CRE-Lox system.	In progress	M 17.5	42
	T 17.9	Use of the RNAi library approach to identify genes involved in the checkpoint response (possibly) activated in stem cells by expression of the NPM mutant or to isolate gene whose inactivation show synthetic lethal interaction with the expression of the NPM mutant	Not yet started	M 17.6	48
	T 17.10	Generation of an ES mouse cell line in which the NPM expression can be knocked down through siRNA in an inducible way	In progress	M 17.7	36
	T 17.11	Analysis of NPM role in DNA damage repair	In progress	M 17.8	42
	T 17.12	Generation of inducible KO mice for the two different NPM isoforms	In progress	M 17.9	48
	T 17.13	Infection of primary cells derived from the above mice with the RNAi library to identify genes that are likely to be responsible for the checkpoint activation induced by the lack of NPM	Not yet started	M 17.10	48

SUMMARY of research activities during 2006

Cluster 4 (WPs 18-20): Ras/Raf Pathway and cellular senescence (GEM, Pasteur, CNIO2 (coordinator))

Work in cluster 4 is directed towards two main objectives, namely to define the role of the Raf pathway in tumourigenesis (GEM) and to identify novel tumour suppressor pathways related to the phenomenon of oncogene-induced senescence (Pasteur & CNIO2).

Novel results from GEM substantiate the requirement for Raf-1 in the maintenance of Ras-induced epithelial tumours and demonstrate that the main process affected by Raf-1 deletion in established tumours is impaired proliferation accompanied by dramatically increased differentiation. In addition, the role of B-Raf in the development of epithelial tumours has been further explored, and the obtained data suggest that B-Raf is necessary to maintain an optimal level of MEK/ERK phosphorylation. Ongoing studies are aimed at shedding light on the effect of B-Raf ablation on the development of insulinomas. Finally, GEM has continued structure/function studies to determine the molecular basis of the interaction between Raf-1 and Rok- α .

Pasteur performed a large scale PML-induced senescence bypass screen with telomerized fibroblasts. The sophisticated screening approach yielded promising candidate genes that might be relevant to PML function and are currently being validated in functional assays (see Figure 1). In a complementary approach, Pasteur has been working on the development of an shRNA sub-library targeted against individual enzymes of the SUMO pathway that, together with the NKI DUB library (directed against de-ubiquitinating enzymes) will be used to screen for genes relevant to PML- and PIASy-induced senescence. In addition, Pasteur has started a collaboration with partner CNIO1, working on an unprecedented role for PIASy in telomere-driven senescence.

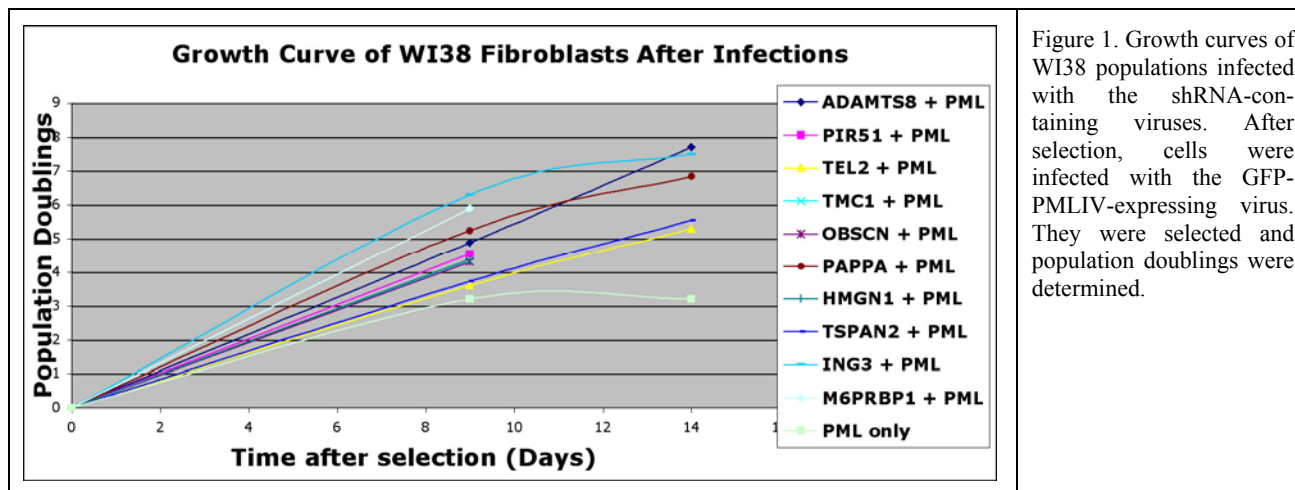


Figure 1. Growth curves of WI38 populations infected with the shRNA-containing viruses. After selection, cells were infected with the GFP-PMLIV-expressing virus. They were selected and population doublings were determined.

Ongoing work of CNIO2 is aimed at optimizing the design of a senescence bypass assay, using telomerized IMR90 human primary fibroblasts carrying estrogen inducible Mek (Mek:ER). As a complementary approach, CNIO2 has developed a strategy to identify novel regulators of key genes involved in OIS using an miRNA library. CNIO2 has further performed a screening of the complete 8k-NKI shRNA library in search of novel inhibitors of the transcription factor E2F1 but, unfortunately the validation process did not confirm any of the candidate genes picked up in this screen.

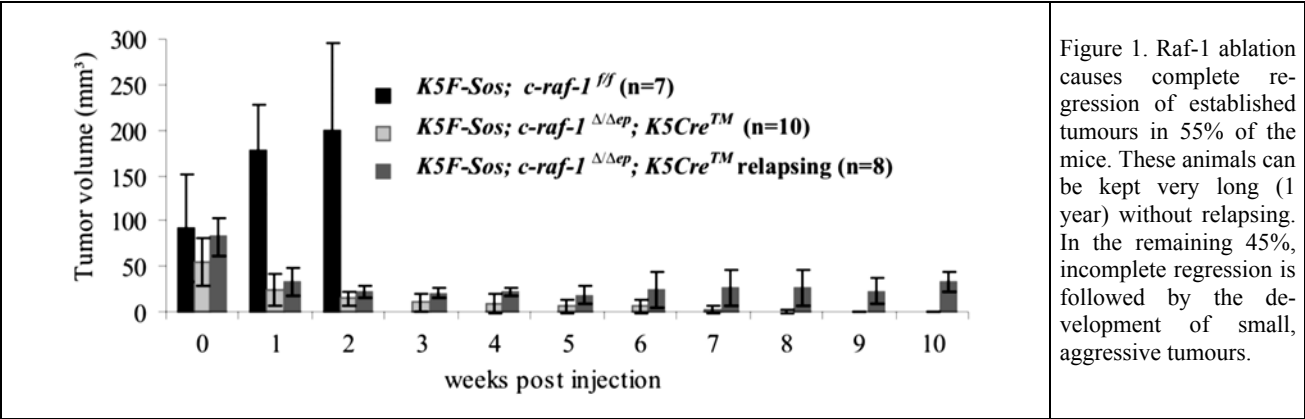
Cluster 4: WP 18
Targets of the Raf pathway in tumourigenesis in vivo and in vitro.
(GEM)

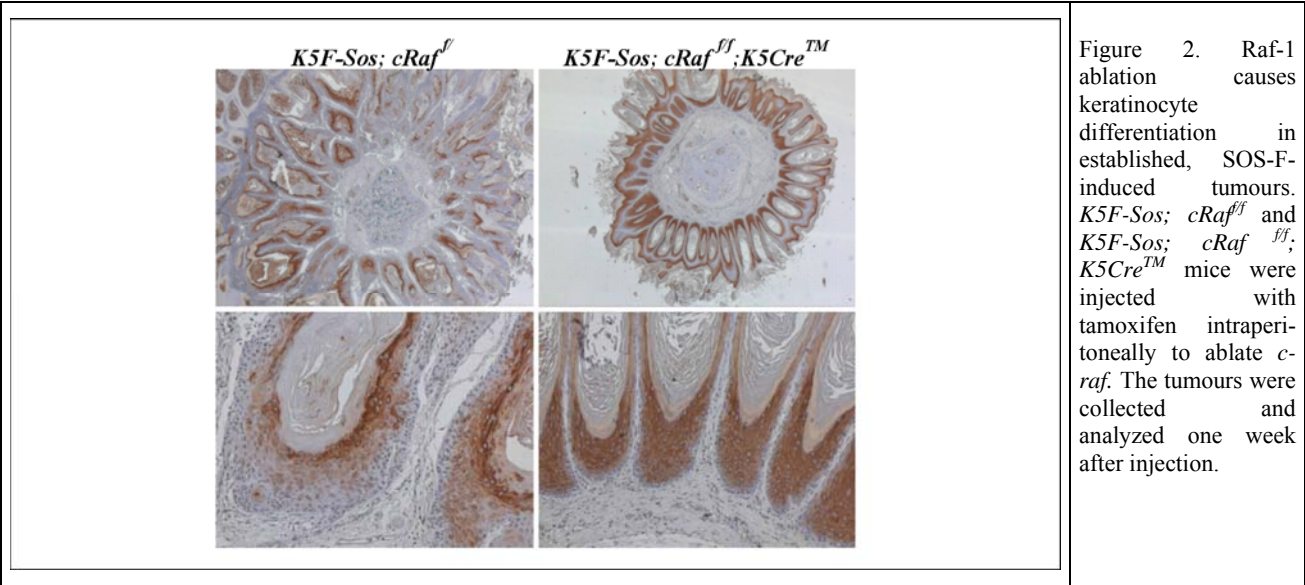
WP 18 uses conventional and conditional gene ablation to identify the molecular mechanisms underlying the unique functions of Raf-1 and B-Raf in apoptosis and to investigate whether two members of the Raf kinase family, B-Raf and Raf-1, are necessary for tumour development. Our aims are: to validate the Raf kinases as therapeutic targets and identify the spectrum of tumours expected to benefit from a therapy targeting Raf; and to identify the Raf-dependent processes as well as the Raf downstream effectors relevant for tumourigenesis. This will be important to direct alternative therapeutic approaches in case the interactions involved should prove to be unsuitable as drug target, or in case targeting them would be associated with intolerable side effects.

We have shown that Raf-1 and, to a lesser extent, B-Raf are essential for the development of Ras-driven epithelial tumours. Strikingly, Raf-1 ablation causes the regression of established tumours; however, about 45% of the mice relapse with tumours that are apparently more malignant than the original tumour. Raf-1 KO keratinocytes are hypersensitive to TGF- β -mediated growth arrest and EMT. Chemical inhibition of the Raf-1-interacting kinase ROK- α reverts both phenotypes. B-Raf, too, plays a yet uncharacterized role in the development of epithelial tumours, and an important role in the development of insulinomas. B-Raf ablation decreases VEGF production in the tumour and causes a dramatic alteration of the tumour stroma, both in terms of stromal cells and matrix deposition. We are focussing on the existing Raf-deficient tumour models, which are being thoroughly characterized.

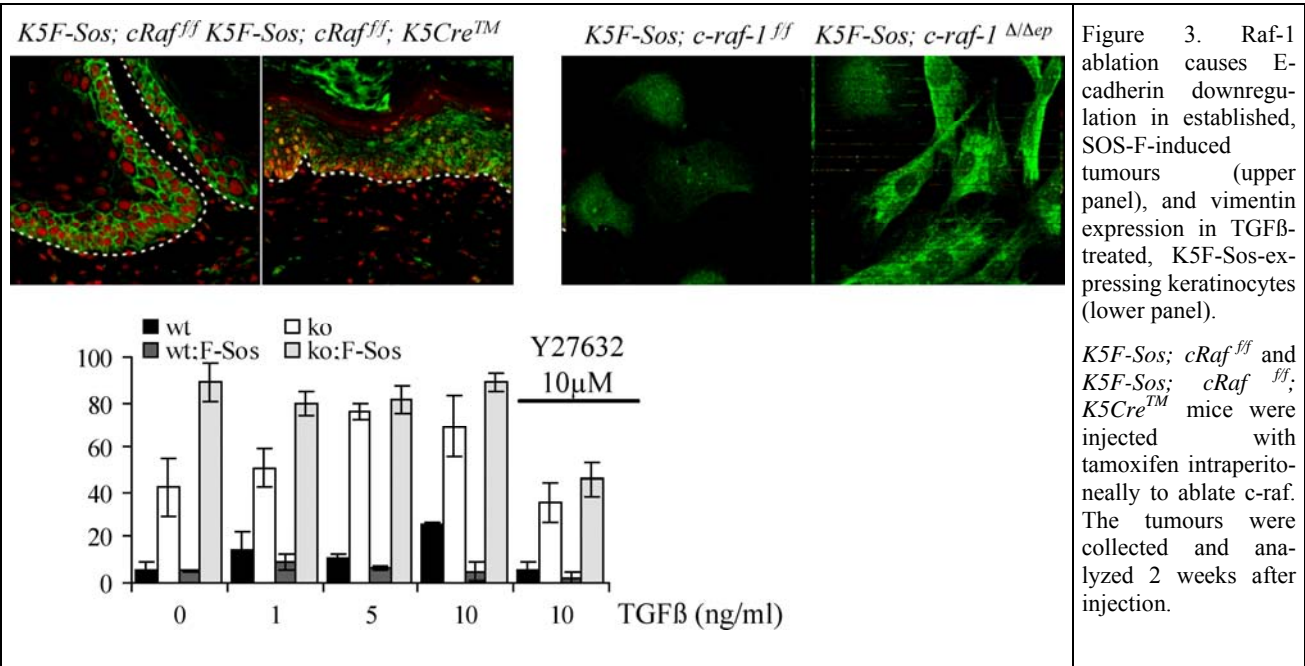
Role of Raf in epithelial carcinogenesis

In 2006, we have collected more data that have substantiated the **requirement for Raf-1 in the maintenance of Ras-induced epithelial tumours** (Figure 1), and we have shown conclusively that the main processes affected by Raf-1 deletion in established tumours are proliferation, which is reduced, and differentiation, which is dramatically increased (Figure 2).





In addition, we have investigated more relapsing tumours and have confirmed that, in line with their more invasive phenotype, E-cadherin expression is slightly less expressed and delocalized, a diagnostic sign of EMT. Furthermore, we have confirmed that Raf-1 KO keratinocytes expressing *K5F-Sos* undergo EMT spontaneously to a certain degree, and massively when exposed to TGF- β .



We are still trying to find a conclusive molecular connection between Raf-1 ablation and the phenotype(s) observed. To this aim, we have been analyzing the response of primary wt and KO keratinocytes, carrying or not the *K5F-Sos* transgene, to TGF- β . The results have not been conclusive so far, and there is a lot of variation among cultures, some of which is due to differences in the confluence of the cells. Clearly, more experiments are needed in this area. We did, however, notice an increase in the phosphorylation of the Rok targets ezrin and JNK, both reduced by chemical inhibition of Rok.

In parallel to the *in vivo* experiments, we are validating the putative targets/processes identified *in vitro*. In particular, we have continued structure/function studies to determine the **basis of the interaction between Raf-1 and Rok- α** . Using site-directed mutagenesis, we have identified a Zn⁺⁺-finger in the regulatory domain of Raf-1 as the structure responsible for its interaction with Rok- α . Interestingly, this structure is extremely similar to that of the autoinhibitory domain of Rok- α itself. In 2006, we have obtained evidence that MEK/ERK control the interaction between Rok- α and Raf-1, likely by phosphorylating Raf-1. We are currently testing Raf-1 phosphorylation mutants to determine which of the ERK phosphorylation sites, if any, play a role in the interaction. We have also been trying to test the hypothesis that Raf-1 works as an inhibitor of Rok- α by binding directly to its catalytic domain. Towards this, we have been expressing different published Raf-1 constructs in bacteria, unfortunately with very limited success due essentially to problems with degradation and/or insolubility of the constructs. Troubleshooting has been painstakingly slow and not very successful, and we are currently trying different strategies to obtain purified Raf-1 from eukaryotic cells and/or *in vitro* translated Raf-1.

The role of **B-Raf in the development of epithelial tumours** has been further confirmed, and we have evidence that *in vivo* the major impact of B-Raf ablation is on proliferation. We have been trying to confirm this *in vitro* in keratinocyte cultures, but again we have encountered problems due to variation among cultures. We will need to increase the number of individual mice tested to be able to make a conclusive statement. Molecularly, there is a consistent trend towards decreased MEK and ERK phosphorylation, strongly suggesting that, in contrast to Raf-1, B-Raf is necessary to maintain an optimal level of MEK/ERK phosphorylation.

To investigate whether the **ablation of both B-Raf and Raf-1** is more effective than the ablation of each kinase by itself in the prevention, and more importantly in the maintenance, of epithelial tumours, we have generated an epidermis-restricted double KO (B-Raf/Raf-1) that shows a severe phenotype prior to tumour induction. This phenotype precludes the use of these animals in a tumour model and will be characterized thoroughly in the context of a separate project.

In 2006, we have advanced significantly the project on the effect of **B-Raf ablation on the development of insulinomas**. We have further characterized the effect of B-Raf ablation on the tumour stroma in the insulinoma model, showing that the KO tumours contain much less myofibroblasts than the wt (Figure 4) and that they produce less TGF- β (not shown). The latter finding needs to be confirmed by analyzing more tumours.

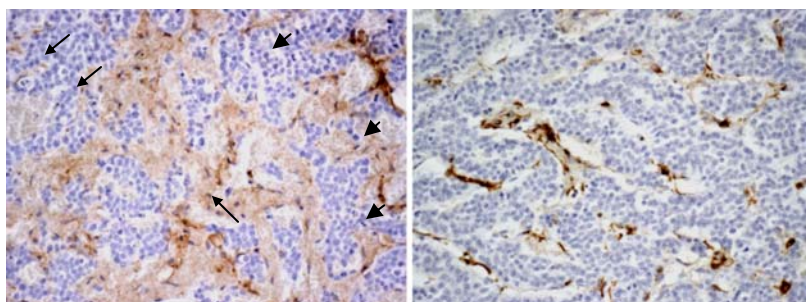


Figure 4. Reduced recruitment of myofibroblasts in B-Raf KO insulinomas. The sections are stained with an antibody against alpha-smooth muscle actin (brown staining), which reacts with both myofibroblasts (arrows) and pericytes (arrowheads). Myofibroblasts are virtually absent in the KO tumours.

In addition, we will analyze cell lines established from WT or B-Raf KO tumours to test whether the decrease in TGF- β production is a cell-autonomous defect. So far, the study of the signalling status of *b-raf*-deficient lines has shown that they contain less pERK and pMEK than wt cell lines; expression of the transcription factor Egr1, an ERK target, is strongly decreased or absent (Figure 5). Interestingly, Egr1 activates the transcription of both the TGF- β and the fibronectin gene. Since both TGF- β production and ECM are reduced in *b-raf*-deficient tumours, this suggests that lack of Egr1 expression maybe the crucial event in the delayed tumour formation/progression observed in B-Raf ablated animals.

Third Annual Periodic Activity Report (2006)

We had planned to test whether silencing Raf by RNAi in human tumour cell lines impinges on the same putative targets/processes identified in the mouse. These experiments are still outstanding, the major difficulty having been the search for an appropriate cell line. The lines described in the literature as TGF- β -sensitive have been so far disappointing. This milestone (M18.8) is therefore delayed. We will continue the search (two more candidates to be tested). In addition, we have encountered problems in knocking down Rok- α in WT or Raf-1 KO primary keratinocytes expressing K5-SOS-F and might have to resort to the exclusive use of the Rok inhibitor to validate the hypothesis that the phenotypes of Raf-1-deficient, SOS-transformed keratinocytes are due to hyperactivation of Rok- α . It has not been possible to obtain human immortalized insulinoma cell lines to test whether B-Raf knock-down in these cells has the same effect as in the mouse insulinoma. This milestone (M18.9) has been cancelled.

Finally, we had planned to prepare constructs for the silencing of Rok- α in the mouse. This task was contingent on the success of the technological development by NK11, which has not, so far, been encouraging. This milestone (M18.10) has been cancelled.

Objectives (months 37-42; revised due to change of strategy)

- Identify Raf-dependent processes in tumourigenesis.
- In-depth analysis of the Raf-dependent processes in tumourigenesis
- Identify the downstream effectors relevant in the context of tumourigenesis.
- Molecular analysis of the interaction between Raf-1 and Rok- α
- Validate the downstream effectors relevant in the context of tumourigenesis.

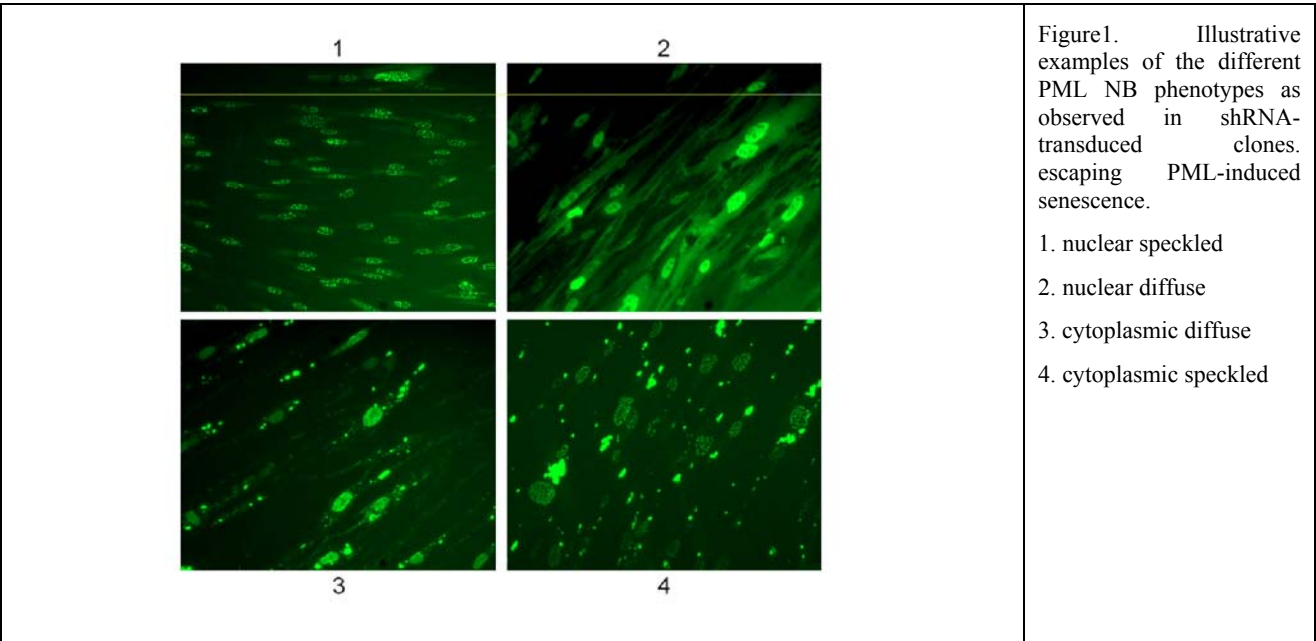
Cumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 18.3	Phenotype analysis of conditional Raf-1 knockout tumour models in vivo in vivo.	achieved/in progress	M 18.1	12
	T 18.4	Phenotype analysis of conditional B-Raf knockout tumour models in vivo in vivo.	achieved/in progress	M 18.2	18
	T 18.5	“Educated guess” approach to define downstream targets of Raf-1 on the basis of the phenotype	achieved/in progress	M 18.3	18
	T 18.6	“Educated guess” approach to define downstream targets of B-Raf on the basis of the phenotype	achieved/in progress	M 18.4	24
Update 12-30	T 18.7	Structure/function studies on the interaction between Raf-1 and its interaction partner Rok- α	achieved/in progress	M 18.5	24
	T 18.8	Validation of the Raf-1 downstream target identified in the first 18 months (Rok- α) by RNAi in vitro, in mouse keratinocytes expressing activated SOS	in progress	M 18.6	30
	T 18.9	Establishment of WT and B-Raf-deficient tumour derived β -cell lines	achieved	M 18.7	20
	T 18.10	Validation of the B-Raf downstream effector identified in the first 18 months (ERK phosphorylation and VEGF production) by RNAi in vitro, tumour derived β -cell lines	in progress	M18.7	27
	T 18.11	Validation of the Raf-1 phenotype and downstream targets in human tumour cell lines	in progress	M18.8	30
	T 18.12	Validation of the B-Raf phenotype and downstream targets in human tumour cell lines	in progress	M18.9	30
	T 18.13	Design and generation of siRNA constructs for the conditional knockdown of Rok- α in the mouse	to be started	M 18.10	30
Update 25-42	T 18.14	Characterize the role of Raf-1 in EMT and metastasis	achieved/in progress	M 18.11	36
	T 18.15	Characterize the role of B-Raf in the development of epithelial tumours	achieved/in progress	M 18.12	42
	T 18.16	Characterize the role of B-Raf in the maintenance of epithelial tumours	to be started	M 18.13	42
	T 18.17	Perform epidermis-restricted double (Raf-1/B-Raf) KO	achieved	M 18.14	33
	T 18.18	Determine the impact of epidermis-restricted double (Raf-1/B-Raf) KO on development and maintenance of epithelial tumours	cancelled	M 18.15	48
	T 18.19	Characterize the effect of B-Raf ablation on the tumour stroma in the insulinoma model	achieved	M 18.16	36
	T 18.20	Monitor the status of tumourigenesis-relevant signalling pathway that cross-talk with, or are downstream of, Raf, by immunohistochemical analysis of in tumour sections and/or biochemically in tumour explants.	in progress	M 18.17	48

Cluster 4: WP 19
Deciphering PML- and PIASy- Induced Senescence
(Pasteur)

Originally, the main objective of WP19 was to characterize novel mediators of PML-induced senescence. To clarify this issue, we initially proposed two strategies, senescence-bypass shRNA screens and reporter cell line based ERMtag screens. However, due to the problems encountered with the ERMtag screening technology, this approach was abandoned and we decided to rather focus on senescence-escape shRNA screens. In the meanwhile, we identified the SUMO E3 ligase PIASy as a potent inducer of senescence and thus decided to extend the screen to both PML and PIASy. We performed a series of pilot experiments as well as a first round of infection at a small scale to optimize screening procedures.

During 2006, we performed a large scale screen including several modifications. First, the starting material of fibroblasts was increased to 6.4×10^7 , second, telomerized fibroblasts were used to extend lifespan of escaping clones, third, to minimize the time for viral infections and therefore to maximize lifespan for clonal expansion of senescence-escaping cells, we performed serial infections with the maximal number of starting cells and fourth, cells were now plated more sparsely on P500 plates. So, a total of 5 rounds of 400 individual infections was applied. The screen yielded a total of 80 cell clones which have been analyzed by immunofluorescence for the PML pattern. If all of them expressed the GFP-PML-IV fusion, clones could be roughly classified into 4 groups according to their PML Nuclear Body (NB) pattern (Figure 1).

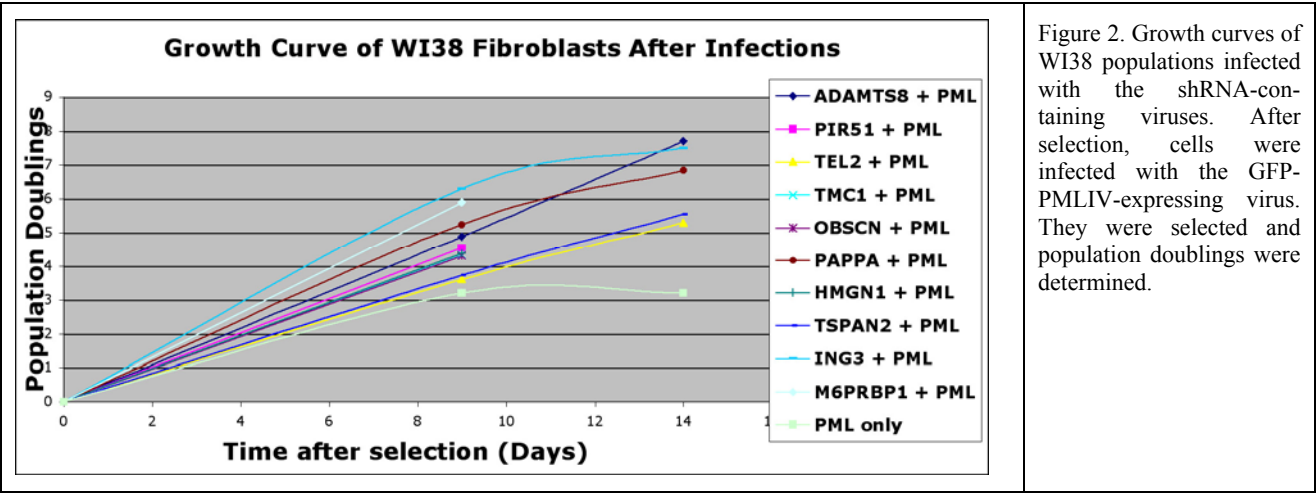


Of the original 80 cell clones that escaped PML-induced senescence, 35 have been analyzed for their shRNA content by sequencing. The number of unrelated shRNAs recovered from each individual cell clone varied from 2 to 15. A number of putative candidate genes were identified and, based on their presence in different cell clones, their abundance within each clone, and their biological relevance, ten of them were selected for validation experiments (Table 1). We cloned individual gene-specific shRNA constructs into pRetro-Super to verify that specific KD of candidates can circumvent PML-induced senescence. Preliminary experiments tend to indicate that five genes (ING3, ADAMTS8, PAPP, TSPAN2 and TEL2) were positive in this first

validation assay (Figure 2). These results need to be confirmed in a second round of infections and using distinct shRNA directed towards these 5 genes. This approach will also be extended to other candidate shRNAs..

PML NB Phenotype	shRNA-target
Normal	PIR51
Normal	TEL2
Normal/Cystolic	PAPPA
Diffused/Cystolic	HMG1
Diffused	ADAMTS8
Diffused	M6PRBP1
Diffused	TMC1
Diffused	TSPAN2
Diffused	ING3
Diffused	OBSCN
Diffused	CBLB
Diffused	SPG3A

Table 1. In parallel, to identify mediators of PML function, we searched for PML interacting proteins as an alternative approach to shRNA screens. This led to identify a physical and functional interaction between PML and the MAR-associated protein SATB1 in regulating chromatin loop architecture (Kumar et al., NCB, in press). Given that the senescent state is accompanied by major changes in chromatin organization, the association between PML and SATB1 is likely to play an important role in PML-driven senescence.



Due to the major task of establishing a screening system that allow us to identify candidate genes involved in PML-induced senescence, the same approach has not yet been used in the context of the PIASy protein. However, to clarify the role of PIASy in senescence, an alternative candidate gene approach was taken. This led to identify PIASy (and its homologue in yeast, namely Plilp) as an important regulator of telomere length (Xhelmace et al., EMBO J, 2005, PNAS, in press; ongoing work in collaboration with M. Blasco, CNIO1).

Third, to study the role of SUMO and SUMO-like modifications in senescence, we started to generate a library of shRNA targeted against individual enzymes of the SUMO pathway and its relatives. This sub-library together with the NKI DUB library (directed against de-ubiquitinating enzymes) will further be used to screen for genes relevant to PML- and PIASy-induced senescence.

Third Annual Periodic Activity Report (2006)

These three complementary approaches will increase our understanding on the still poorly explored role of the PML/PIASy/SUMO network in oncogene-driven senescence and hopefully yield novel targets relevant to cancer therapy.

Objectives (revised due to change of strategy)

- Identify and characterize mediators of PML-induced senescence
- Identify and characterize mediators of PIASy-induced senescence
- Define the role of SUMO and SUMO-like modifications in PML- and PIASy-induced senescence

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 19.1	Bar code screening of human primary fibroblasts co-infected by the high-titer retroviral RNAi libraries and PML.	Achieved	M 19.3	18
	T 19.2	Generation of human primary fibroblast cell lines that carry a GFP reporter for the PML gene.	Cancelled	M 19.1	12
	T 19.3	Generation of human primary fibroblasts expressing the tet-repressor in addition to the PML-GFP reporter.	Cancelled	M 19.1	12
	T 19.4	Screening of the PML-GFP cell lines using high-titer RNAi libraries.	Cancelled	M 19.2	18
	T 19.5	Screening of tet-repressor cell lines expressing PML-GFP using the ERM tag retroviral vector.	Cancelled	M 19.2	18
	T 19.6	Identification of relevant genes by database analysis and ISH-based expression studies. Comparison to the other genes identified in the screens proposed in other work packages (p53, pBB, RAF).	Not started yet	M 19.4 M19.5	30
Update 12-30	T 19.7	Bar code screening of human primary fibroblasts co-infected by the high-titer retroviral shRNA libraries and PML.	Achieved	M19.6	36
	T 19.8	Bar code screening of human primary fibroblasts co-infected by the high-titer retroviral RNAi libraries and PIASy.	On hold until feasibility is proven by T19.7	M 19.7	48
	T 19.9	Generation of a shRNA sub-library from the NKI library targeting enzymes involved in SUMO and SUMO-like modifications	In progress	M 19.8	42
	T 19.10	Clone-by-clone senescence bypass screen of PML- and PIASy-overexpressing human fibroblasts with the SUMO sub-library	To be started	M 19.8	48
	T 19.11	Identification and characterization of relevant genes identified in tasks 7, 8 and 10 by database analysis, expression studies in tumours, and comparison with candidate genes identified in complementary screens performed in other WPs (p53, RAF, Rb)	To be started	M 19.9	48
Update 25-42		Due to the delay in the realization of the tasks planned for months 1-30, no additional items have been included into this update for months 25-42			

Cluster 4: WP 20

Identification of novel tumour suppressor pathways (CNIO2)

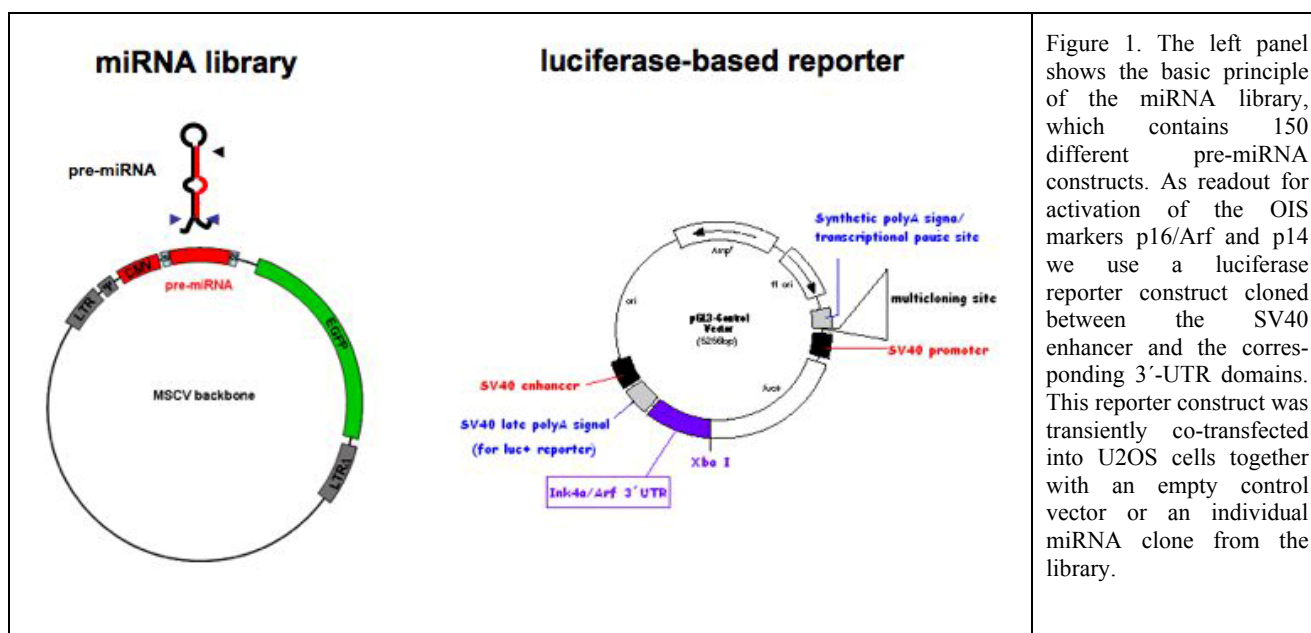
The global goal of this work package is the identification of novel tumour suppressor pathways using the following three different experimental approaches:

A) *Oncogenomics of the stepwise neoplastic transformation of human cells in culture*

Recently, we have identified and validated various markers for oncogene-induced senescence (OIS) and used some of these markers to proof that OIS occurs in vivo.

To address the issue whether the identified OIS markers are functionally implicated in the process of OIS, we have generated a complete RNAi library directed against the entire panel of 50 OIS markers. For this purpose, the corresponding shRNA inserts (3 for each of the 50 OIS markers) were cloned into a retroviral vector conferring hygromycin resistance. Ongoing work is aimed at optimizing the design of a senescence bypass assay, using telomerized IMR90 human primary fibroblasts carrying estrogen inducible Mek alone (Mek:ER). In this model system, 4-hydroxytamoxifen (4OHT)-triggered activation of Mek leads to a permanent growth arrest (OIS), which is supposed to be bypassed upon RNAi directed against one or several OIS markers.

In addition, we have developed a strategy to identify novel regulators of key genes involved in OIS using an miRNA library, which has become available in our laboratory recently (a generous gift from Greg Hannon at Cold Spring Harbor Laboratories). We have already performed initial screenings using reporter constructs carrying 3'-UTR regions of the OIS mediators p16/Arf and p14 (Figure 1). We were able to identify miRNAs inducing a >50% reduction of luciferase activity of both the p16/Arf and p14 reporter constructs. Currently we are validating these miRNAs and their putative target genes in functional assays.



B) shRNA screening for novel regulators of E2F1

Recently, we successfully terminated a complete screening of the 8k-NKI shRNA library on a one-by-one basis and identified novel antagonists of p53 function (see WP10).

Using the same approach, we have performed a screening of the complete 8k-NKI shRNA library in search of novel inhibitors of the transcription factor E2F1. For the screening, we used U2OS cells transiently transfected with the human E2F1 promoter driving expression of luciferase. The E2F1 promoter, which contains potential binding sites for E2F, Sp1, Atf, E4F, NF-kB, as well as CAAT boxes provided an excellent signal-noise ratio, i.e. turned out to be suitable for the screening. A primary screen of the library yielded 15 candidate genes that activated or repressed the promoter >3-fold. Thorough validation of these shRNAs, however, did not confirm any of these candidate genes as efficient activator/repressor of the E2F promoter (Figure 1). In the light of these negative results, the E2F1 screening approach has been abandoned.

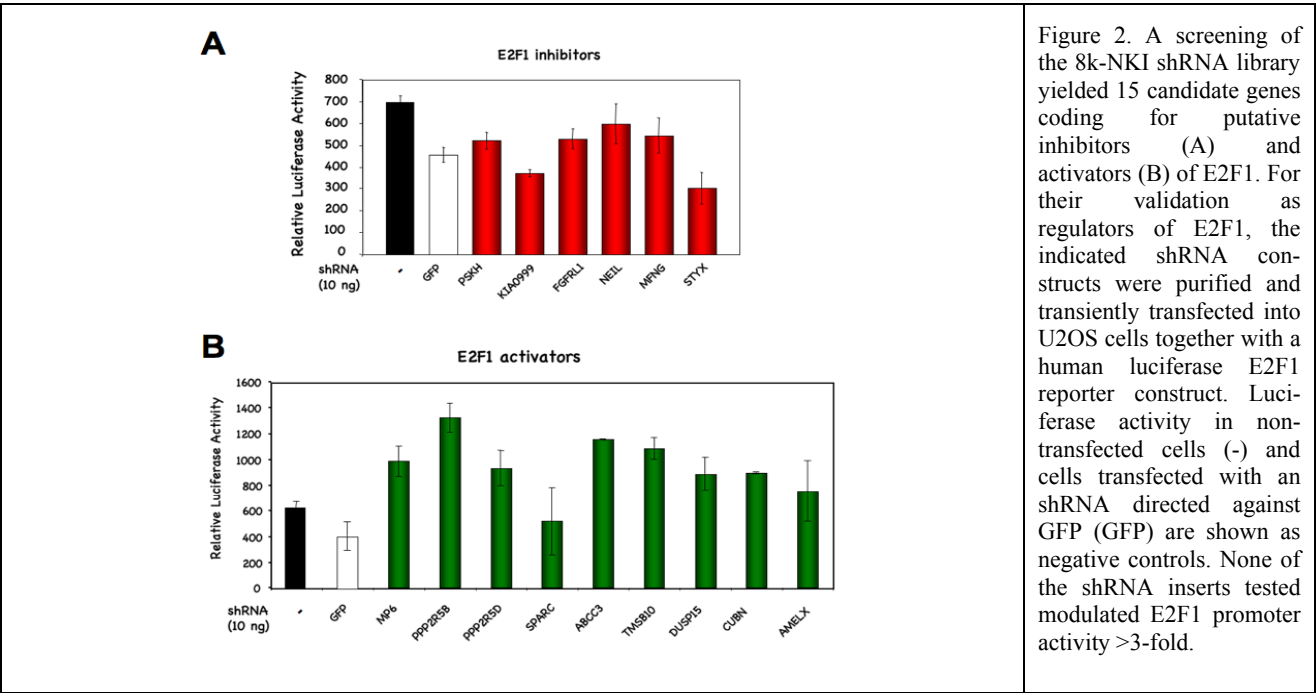


Figure 2. A screening of the 8k-NKI shRNA library yielded 15 candidate genes coding for putative inhibitors (A) and activators (B) of E2F1. For their validation as regulators of E2F1, the indicated shRNA constructs were purified and transiently transfected into U2OS cells together with a human luciferase E2F1 reporter construct. Luciferase activity in non-transfected cells (-) and cells transfected with an shRNA directed against GFP (GFP) are shown as negative controls. None of the shRNA inserts tested modulated E2F1 promoter activity >3-fold.

C) RNAi-induced epigenetic silencing of the tumour suppressor locus *Ink4a/ARF*

Recently, we could show that RNAi-induced heterochromatinization of a locus control region and, at the same time, origin of replication in the tumour suppressor locus *Ink4a/Arf* resulted in transcriptional silencing of the entire locus, i.e. abolished the expression of the tumour suppressors and OIS markers p15^{INK4b}, p16^{INK4a}, and ARF.

To further explore this unprecedented link between RNAi-directed heterochromatinization and tumour suppression we have generated a library of RNAi's covering the entire locus and started to perform ChIP analysis (H3K9) to identify regions susceptible to RNAi-induced silencing.

Objectives

- Understand the functional relevance of OIS markers to the entry of cells into OIS
- Identify novel negative regulators of E2F1
- Understand RNAi-induced epigenetic silencing of the tumour suppressor locus *INK4a/ARF*

Third Annual Periodic Activity Report (2006)

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 20.1	Generation of IMR90 cells stably expressing hTERT and Mek fused to ER alone or in combination with E6/E7 and/or ST by retroviral transduction and subsequent selection	Achieved	M 20.1	6
	T 20.2	Characterization of the growth properties of these cell lines	Achieved	M 20.1	6
	T 20.3	Microarray hybridization using total RNA from tamoxifen-treated IMR90-derived cells and bioinformatic analysis of the data	Achieved	M 20.2	9
	T 20.4	Validation of candidate genes using alternative techniques (RT-PCR, northern-blot, others)	Achieved	M 20.3	12
	T 20.5	Functional assays to discriminate between executors and markers of each step	Broken down to T 20.8 and T 20.9	M 20.3	12
	T 20.6	Use of candidate OIS markers to prove the occurrence of OIS in vivo with animal models	Achieved	M 20.4	18
	T 20.7	Generation of new mouse models of tumourigenesis	Achieved	M 20.5	18
Update 12-30	T 20.8	Generation of shRNA vectors to interfere with the expression of previously identified OIS markers in human and murine cells	Achieved	M 20.6	18
	T 20.9	Characterization of the effect of knocking down OIS on entry into senescence and neoplastic transformation in a model of stepwise neoplastic transformation of human cells in culture	To be started	M 20.7	30
Update 25-42	T 20.10	Create suitable reporter cell system for an shRNA screen aimed at the identification of novel negative regulators of E2F1	Terminated	M 20. 8	33
	T 20.11	shRNA screen for novel negative regulators of E2F1	Terminated	M 20. 8	33
	T 20.12	Validation, and characterization of genes identified in the screen for novel negative regulators of E2F1	Terminated Cancelled	M 20. 9	45
	T 20.13	Generation of RNAi library against non-coding regions of the INK4a/ARF locus	In progress	M 20. 10	36
	T 20.14	Screening of the RNAi library from T 20.13 against non-coding regions of the INK4a/ARF locus using ChIP analysis of heterochromatin marks as read-out	To be started	M 20. 10	36
	T 20.15	Characterization of the regions in the INK4a/ARF locus mediating RNAi-induced epigenetic silencing	To be started	M 20. 8	48

SUMMARY of research activities during 2006
Cluster 5 (WPs 21-23): The MYC Pathway (UNIMAR, ISREC1
(coordinator))

With three projects this cluster aims

1. To screen for novel regulators of the Myc/Miz1 pathway in human epithelial carcinoma cells, aiming to identify novel drug targets to treat Myc driven tumors (UNIMAR),
2. To characterize murine hematopoietic stem cells (HSC) carrying mutations in the Pten pathways (ISREC1), and
3. To identify modulators that increase HSC self-renewal in cooperation with Myc and Pten, as well as identify leukemic stem cells (ISREC1).

Two shRNA-based screens have been completed in this cluster. In the first screen two genes (RAD1 and IRS4), which overcome a p21^{CIP1} mediated G1 growth arrest of colon carcinoma cells in response to low dose UV-irradiation have been identified. UNIMAR demonstrated that upon UV irradiation Miz1 strongly enhances ATR signaling via stabilization of Topbp1. This leads to enhanced levels of Chk1 and p53 phosphorylation. Most interestingly, knockdown of RAD1 reverts these effects identifying Rad1 as a novel essential regulator of the Miz1-TOPBP-Chk1-p53 pathway. In the second screen, UNIMAR performed a barcode screen aimed at identifying genes rescuing the pro-apoptotic effects of MycER expression in osteosarcoma cells. This screen turned out to be remarkably successful and 91 putative candidate genes have been identified. In addition to the identification of genes previously shown to be required for Myc- induced apoptosis, novel genes have also been identified. One of these is Usp28, an ubiquitin-specific protease which seems to antagonize ubiquitin ligases. UNIMAR could show that Usp28 stabilizes the Myc protein and binds to Myc via Fbw7. Most interestingly, depletion of Usp28 causes a Myc mediated cell cycle arrest. These findings now open the exciting possibility that inhibition of Usp28 i.e. by small molecule inhibitors may be effective for therapeutic treatment of Myc driven tumors. Finally, UNIMAR identified Aurora-kinase A (AURKA) as a component essential to maintain proliferation of neuroblastoma cells carrying amplified MYCN. Preliminary analysis indicates that AURKA has two functions in MYCN amplified neuroblastoma cells: Restriction of p53 function and enhancement of MYCN translation.

In the third WP, ISREC1 demonstrated that loss of the Pten gene in mouse HSCs causes CD4⁺ T cell leukemias, suggesting that Pten acts as a tumor suppressor in blood cells. In addition, it was shown that Pten restricts the *in vivo* expression of the HSC-mobilizing cytokine G-CSF causing Pten deficient HSCs to re-localize from the bone marrow to the spleen. In an effort to study a possible cooperation between the Myc and Pten-PI3-kinase pathway, a novel lentiviral system that allows the inducible overexpression or knock-down of any gene of interest in hematopoietic and leukemic cells *in vitro* and *in vivo* has been established. In the future this system will be used to test the activity of various Myc mutants for their ability to affect Pten deficient normal and leukemic HSCs. In a further effort to demonstrate oncogene cooperativity *in vivo*, the ability of endogenous c-Myc to repress p21^{cip1} expression was shown to be essential for activated Ras mediated skin tumorigenesis.

In summary, the cluster has provided significant new insights into the functions of Myc and Pten in normal and transformed cells, and most excitingly has identified a novel putative drug target (Usp28), which could be used for the treatment of Myc driven tumors. During 2006 the cluster has published one paper and has submitted two others.

Cluster 5: WP 21
Targeting the Myc/Miz1 pathway using RNAi and ERMtag-based screens (UNIMAR)

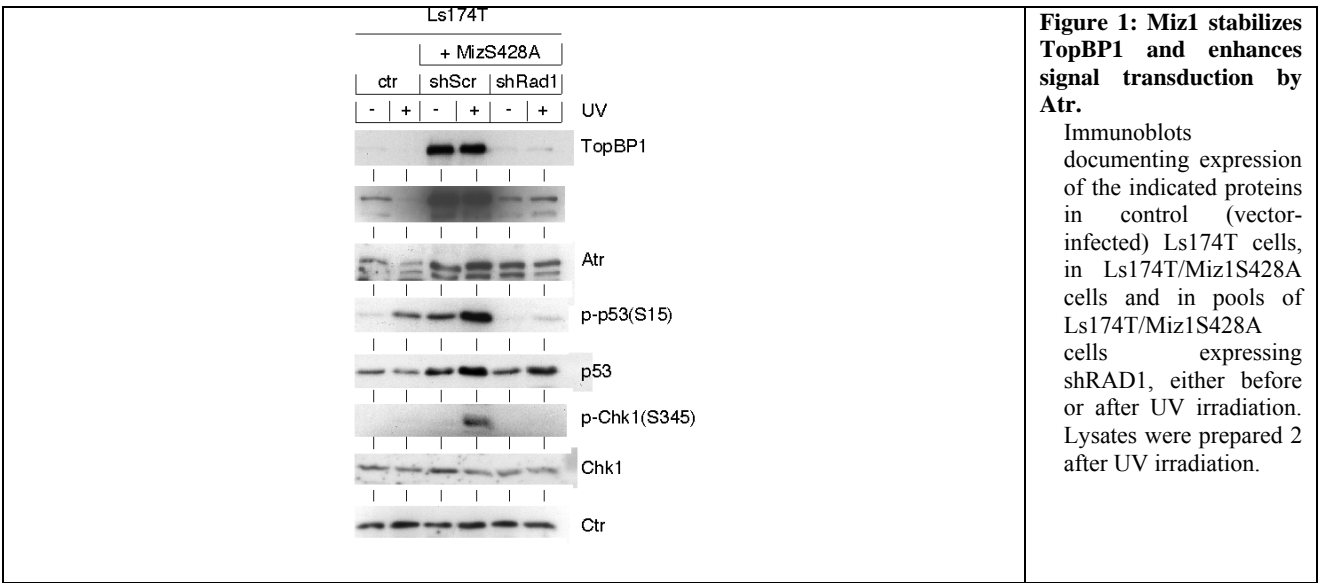
We have reported previously that we have completed two screens in this work package that aim at finding new genes that are required for Miz1 function and for Myc function. Both screens are finished and we are currently completing the biochemical analysis of a first series of hits.

1) In the first screen, we had sensitized colon carcinoma Ls174T to UV irradiation by expression of a mutant allele of Miz1 that cannot be phosphorylated by Akt (Miz1S428A). Such cells undergo a permanent cell cycle arrest in response to low dose UV irradiation and we can use this phenotype to screen for shRNAs that allow them to rapidly resume proliferation as control cells do. The molecular analysis shows that cells expressing Miz1S428A express high basal levels of p21Cip1 and that they strongly suppress expression of *CDC25C* mRNA; both can be used as markers for the effects of Miz1.

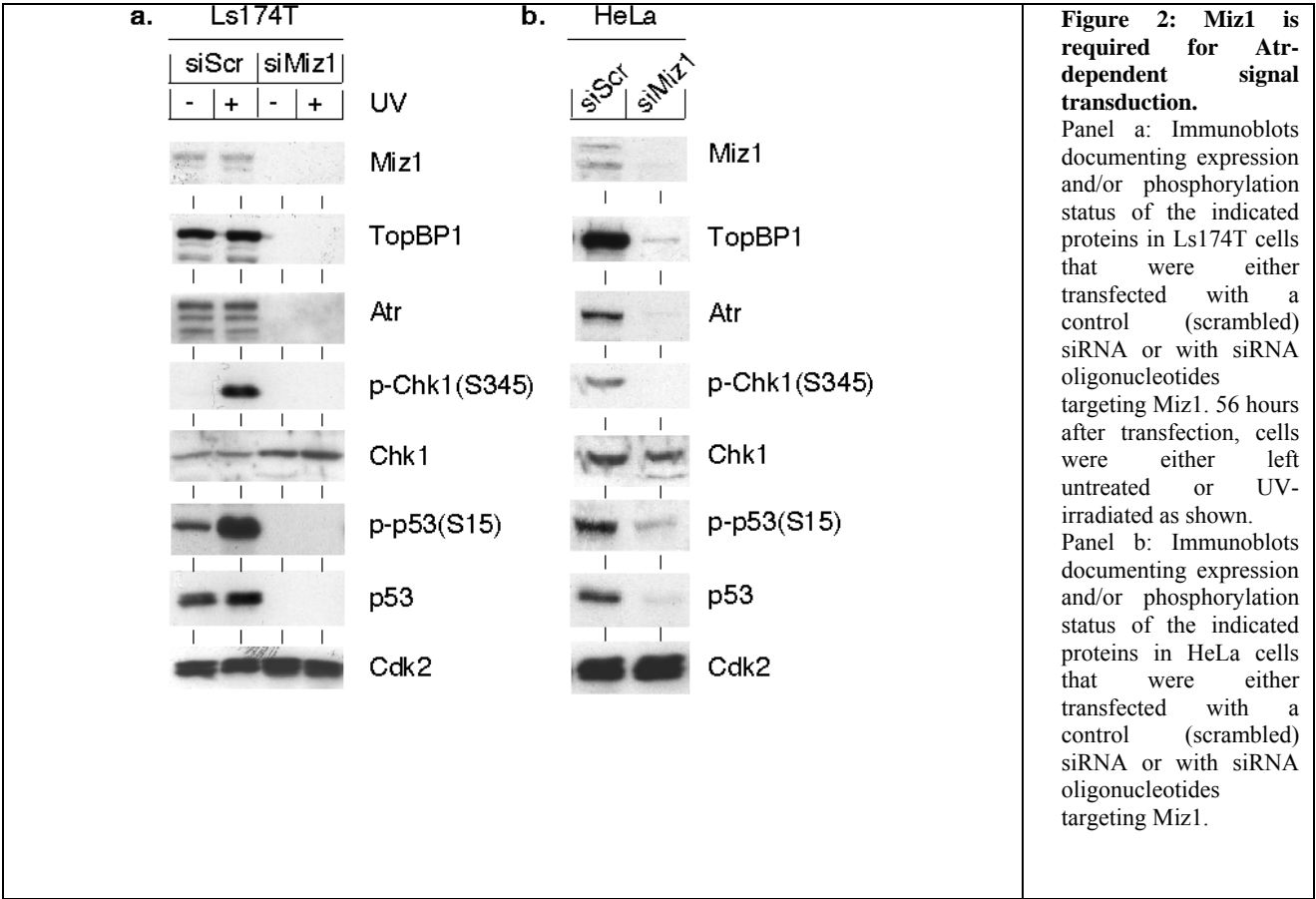
The initial screen yielded multiple hits, which were filtered using restoration of p21Cip1 and *CDC25C* expression to control levels as secondary screens; after this, shRNAs targeting only two genes gave consistent results: those targeting *RAD1* and those targeting *IRS4*. A third gene, which looked promising (Parp2), could not be reproduced using a second shRNA. However, independent work shows that Miz1 is poly-ADPribosylated after UV irradiation, so this could still be a relevant finding (F. Hänel, personal communication). Irs4 has recently been identified in a proteomic analysis as a Myc-associated protein (H. Hermeking, personal communication); independently, we have found Irs4 in a proteomic analysis of a highly enriched preparation of Miz1, so we have now begun to work on this protein.

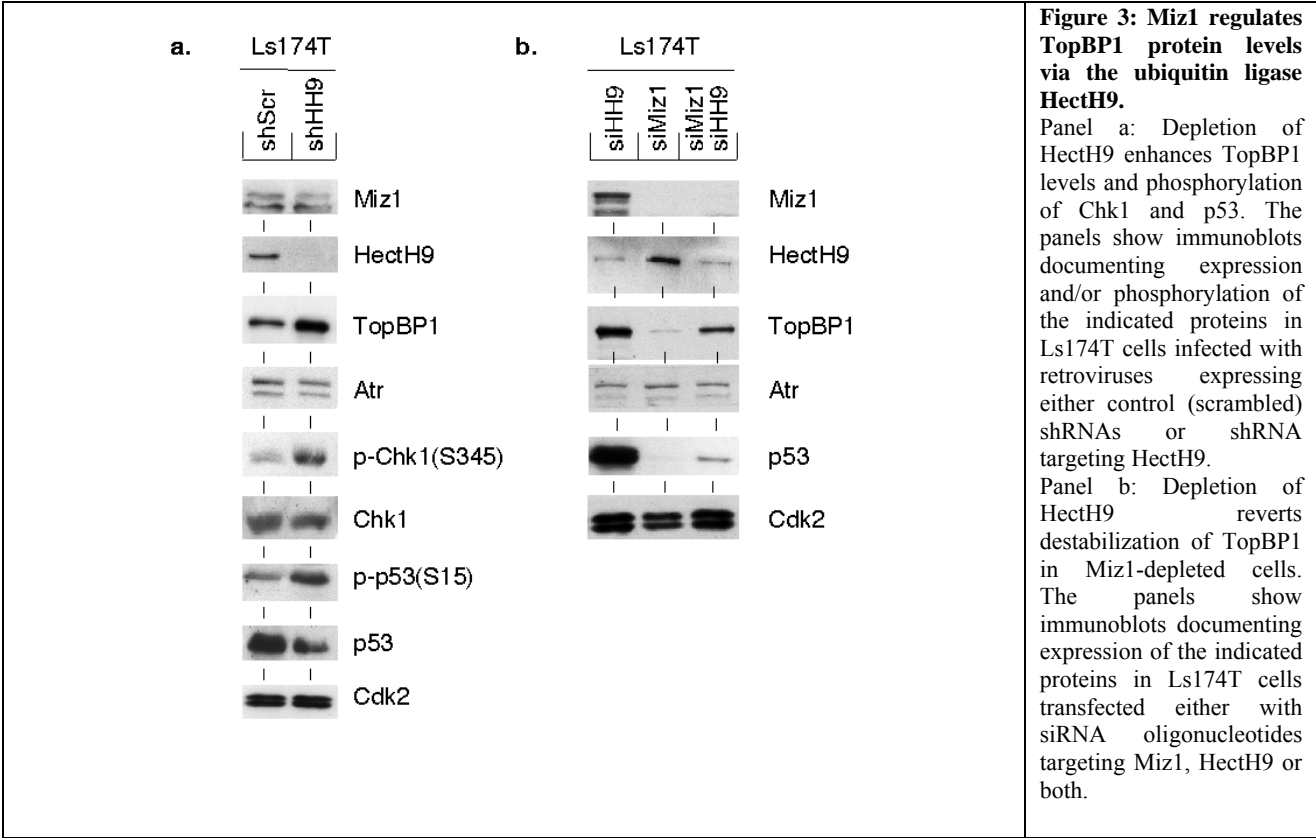
Most of our work has focused on characterizing the interaction of Miz1 with Rad1 in the UV response. Our main findings are:

1. Expression of Miz1 strongly enhances signal transduction by Atr, leading to enhanced levels of Chk1 and p53 phosphorylation upon UV irradiation. shRNAs targeting Rad1 revert these effects. We had previously shown that Miz1 binds to TopBP1, an activator of the Atr kinase. Consistent with both observations, Miz1 strongly enhances steady state levels of Topbp1 and stabilizes Topbp1 against proteasomal degradation (Figure 1). Miz1 does not affect mRNA levels encoding any of the known components of the Atr checkpoint.



- 2. Conversely, depletion of Miz1 leads to a strong decrease in steady state levels of Topbp1 and – to a lesser degree- of Atr and to a loss of Atr-dependent signal transduction. This occurs in the absence of detectable changes in mRNA levels of checkpoint components (Figure 2).
- 3. Myc antagonizes the stabilizing function of Miz1; specifically, depletion of Myc enhances steady state levels of Topbp1 and signaling via the Atr checkpoint..
- 4. Degradation of Topbp1- and in part also of p53- in the absence of Miz1 is mediated by HectH9 (Figure 3). HectH9 ubiquitinates Topbp1 *in vivo* and a point mutant of Topbp1, which is not bound to Miz1, is more ubiquitinated more efficiently by HectH9.



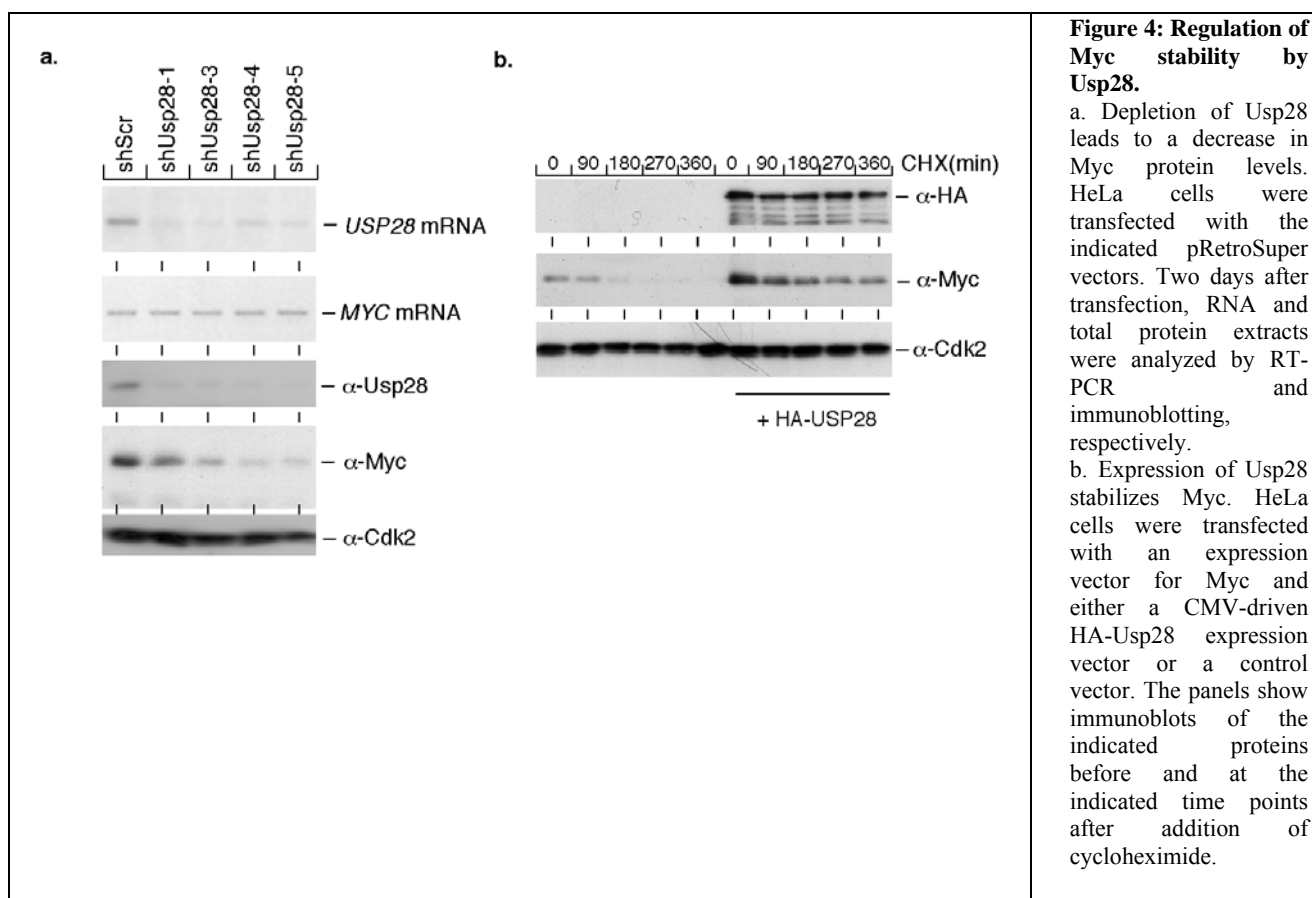


We had previously characterized HectH9 (also called Arf-Bp1) as an ubiquitin E3-ligase that is inhibited by Miz1 and work by Wei Gu had shown that HectH9 can degrade p53. Our data here suggest that inhibition of HectH9 by Miz1 may be an essential component of the UV response. In support of this view, we find that there is sequential degradation of Myc (which would enhance checkpoint signaling) followed a few hours later by degradation of Miz1 (which might terminate the signal) after low-dose UV irradiation. We would also suggest that Miz1 and Myc act upstream of HectH9 to regulate the function of the Atr-Chk1 DNA damage response pathway. The findings also suggest that Myc and Miz1 may have non-transcriptional readouts.

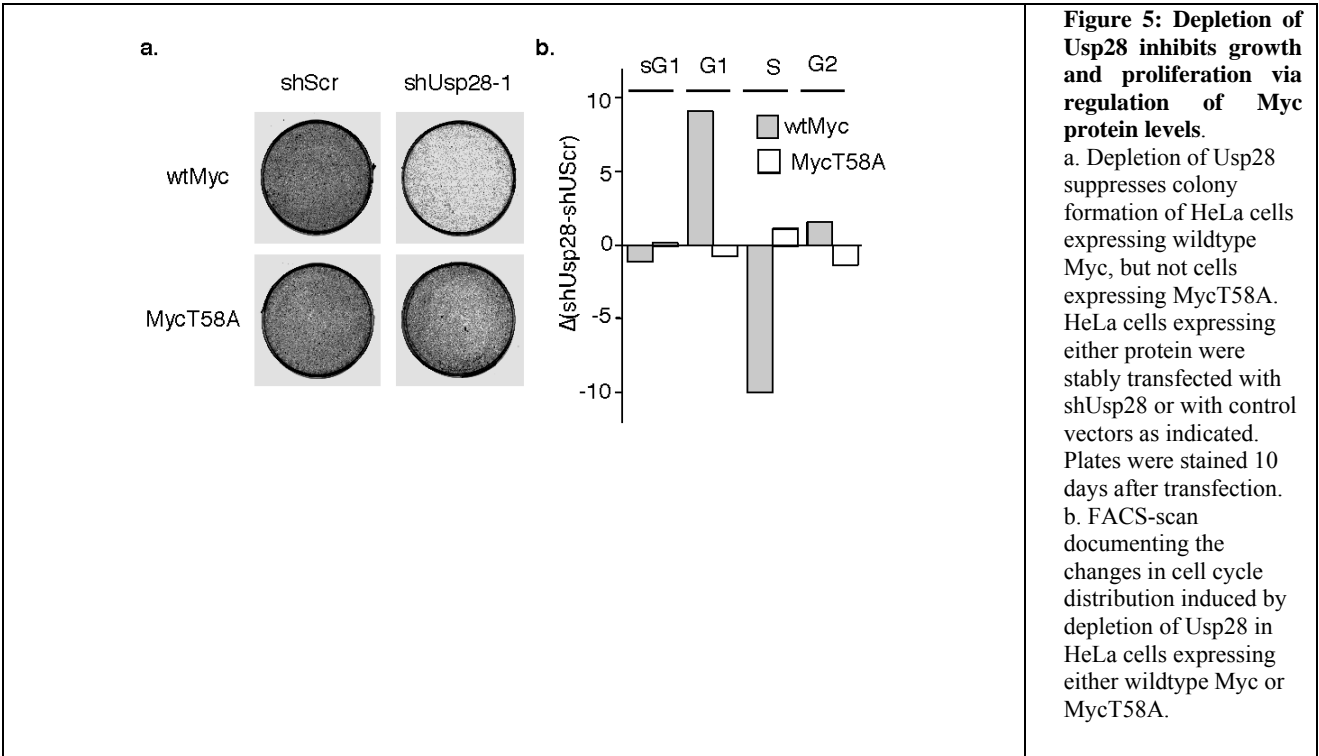
We are currently revising a manuscript describing these findings and try to establish the role of Miz1-mediated stabilization of Topbp1 during the physiological response to UV irradiation.

2) In the second screen, we had used U2OS cells that express a MycER protein and had looked for shRNAs that inhibit Myc-induced apoptosis using a barcode approach. This screen had identified a total of 91 genes that were targeted reproducibly in multiple screens that we carried out. We have begun to characterize several of the hits in detail. Our main focus was on completing the analysis of Usp28 function. We had identified Usp28, since shRNAs targeting Usp28 reduced Myc-induced apoptosis and also reduced levels of both the MycER protein and the endogenous Myc protein in U2OS cells, suggesting that Usp28 (which is a ubiquitin-specific protease and might antagonize the action of ubiquitin ligases) might stabilize Myc. Our main findings are:

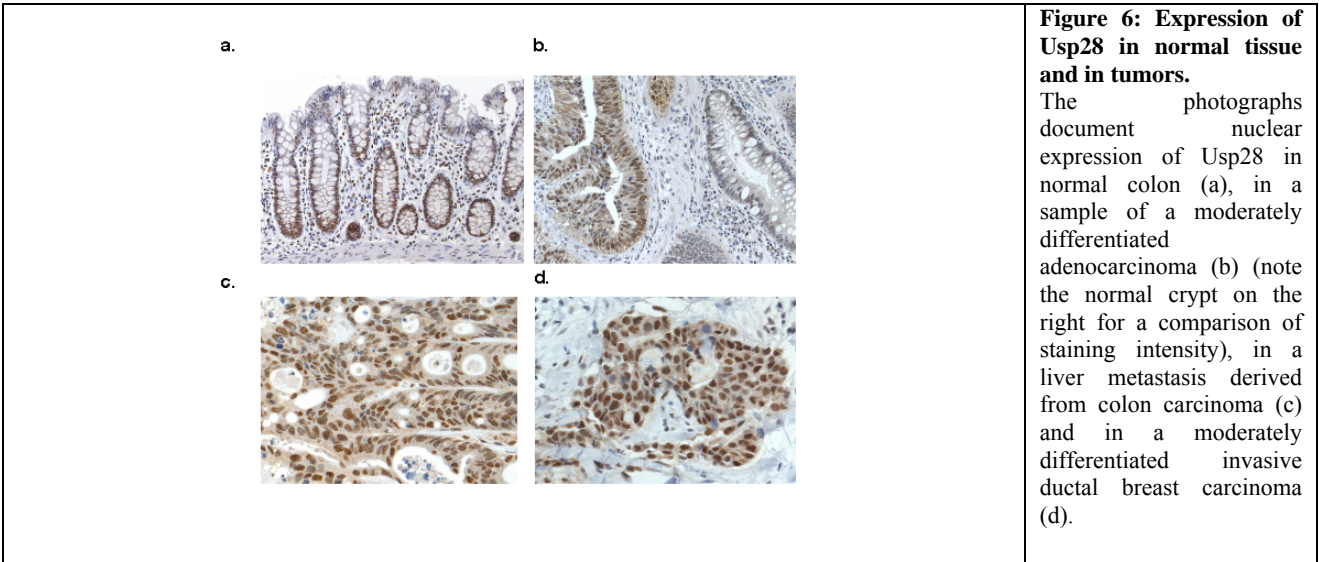
1. Depletion of Usp28 by multiple different shRNAs destabilizes Myc in all human tumor cell lines that we tested. Conversely, ectopic expression of Usp28 stabilizes Myc (Figure 4)



2. Usp28 de-ubiquitinates Myc *in vivo* and *in vitro*.
3. Usp28 specifically antagonizes the action of one ubiquitin ligase of Myc, Fbw7. A point mutation of the Fbw7 recognition site in Myc or depletion of Fbw7 abolishes the effects of either Usp28 or of shRNAs targeting Usp28 on Myc stability. Usp28 also stabilizes a second substrate of Fbw7, cyclin E and again a single point mutant in the Fbw7 binding site abolishes the effect of Usp28.
4. Usp28 does not bind to Myc directly; rather, it binds to Myc via Fbw7. Interestingly, it only binds to Myc via Fbw7 α , one of the three isoforms of Fbw7. Fbw7 α is localized in the nucleus, but is excluded from the nucleolus. In contrast, Fbw7 γ , which is localized in the nucleolus, does not bind to Usp28. We believe that these observations may explain the fact that Myc is selectively degraded in the nucleolus.
5. Depletion of Usp28 arrests proliferation of multiple human tumor cell lines. In HeLa cells, expression of a mutant Myc that is not degraded by Fbw7, but not of wtMyc, fully rescues the growth arrest that is induced by shRNAs targeting Usp28 (Figure 2).



6. Microarray analyses show that shRNAs targeting Usp28 regulate a set of genes that is largely identical to genes that are regulated in response to depletion of Myc.
7. In normal human breast, Usp28 is expressed at low levels. In normal colon, Usp28 is expressed in graded manner with moderate expression in the crypts and undetectable expression at the surface of the epithelium. In both breast and colon tumor samples, expression of Usp28 is highly up-regulated (Figure 3).



A manuscript describing these findings is currently under review. Since Usp28 is a cysteine protease and since cysteine proteases can be inhibited with small molecules, we believe that the identification of its essential role in stabilizing Myc may open the possibility to target Myc therapeutically using small molecules. In order to provide proof-of-principle for this concept, we are currently co-operating with Michel

Third Annual Periodic Activity Report (2006)

Aguet (ISREC) to generate inducible lentiviral shRNA vectors that allow us to regulate levels of Usp28 in xenograft models of human colon tumors.

In addition, we have continued our work on H2a.z; we had identified shRNAs targeting H2a.z several times in the screen and have confirmed that they indeed suppress Myc-induced apoptosis. We found this interesting, since Myc is known to associate with p400 (Domino), a potential exchange factor for H2a.z. Since then, we have found in chromatin-immunoprecipitation assays that activation of Myc induces a rapid exchange of H2a.z at nucleosomes that contain E-boxes that are bound by Myc *in vivo*. Microarray analyses show that depletion of H2a.z strongly inhibits both activation and - very surprisingly- gene repression of multiple target genes by Myc. We are currently completing this biochemical analysis. We will focus on this mechanistic analysis in the remaining funding period rather than expanding our analysis into the developmental control of apoptosis.

Objectives (revised due to change of strategy)

- Analysis of role of H2a.z exchange in Myc-dependent gene regulation.

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 30)

	No.	Task name	Status	Associated milestone	Milestone date
Original	T21.1	Reisolate shRNA sequences from positive pools in U2OS-MycER screen and sequence insert (as barcode)	Achieved	M 21.2	6
	T21.2	Reisolate shRNA sequences from positive pools in Ls147T-Miz1S428A screen and sequence inserts	Achieved	M 21.1	6
	T21.3	Design and carry out strategies to identify positive insert from U2OS-MycER screen.	Achieved	M 21.5	12
	T21.4	Same as Task 3 for Ls147T-Miz1S428A screen.	Achieved	M 21.3	12
	T21.5	Functional tests of positive inserts. Test of control shRNAs from MycER screen.	Achieved (and still ongoing)	M 21.5	18
	T21.6	Functional tests of positive inserts. Test of control shRNAs from Miz1S428A screen.	Achieved (and still ongoing)	M 21.3	30
	T21.7	The identified genes will be compared with the genes obtained in the screens in the other work packages.	Achieved (and still ongoing)	M 21.3/.5	30
	T21.8	The expression of the identified genes in primary tumours will be analysed by database analysis and screening of multiple tumour arrays by ISH.	Achieved for Usp28	M 21.4	30
	T21.9	Gene copy numbers for selected overexpressed or non-expressed genes will be determined by FISH	not started yet	M 21.4	
Update 12-30	T 21.10	Biochemical Characterization of Parp2 and Rad1	Achieved (and still ongoing)	M 21.7	36
	T 21.11	Biochemical Characterization of Usp28	Achieved	M 21.6	36
	T 21.12	Biochemical Characterization of Co-Factors (H2a.z) of Myc	In progress	M 21.9	48
	T 21.13	Development of novel assay systems for developmental control of Myc-induced apoptosis	Not started yet	M 21.8	42
Update 25-42	T21.14	Inducible Knockdown of Usp28 in xenograft model (together with ISREC)	In progress	M21.10	48

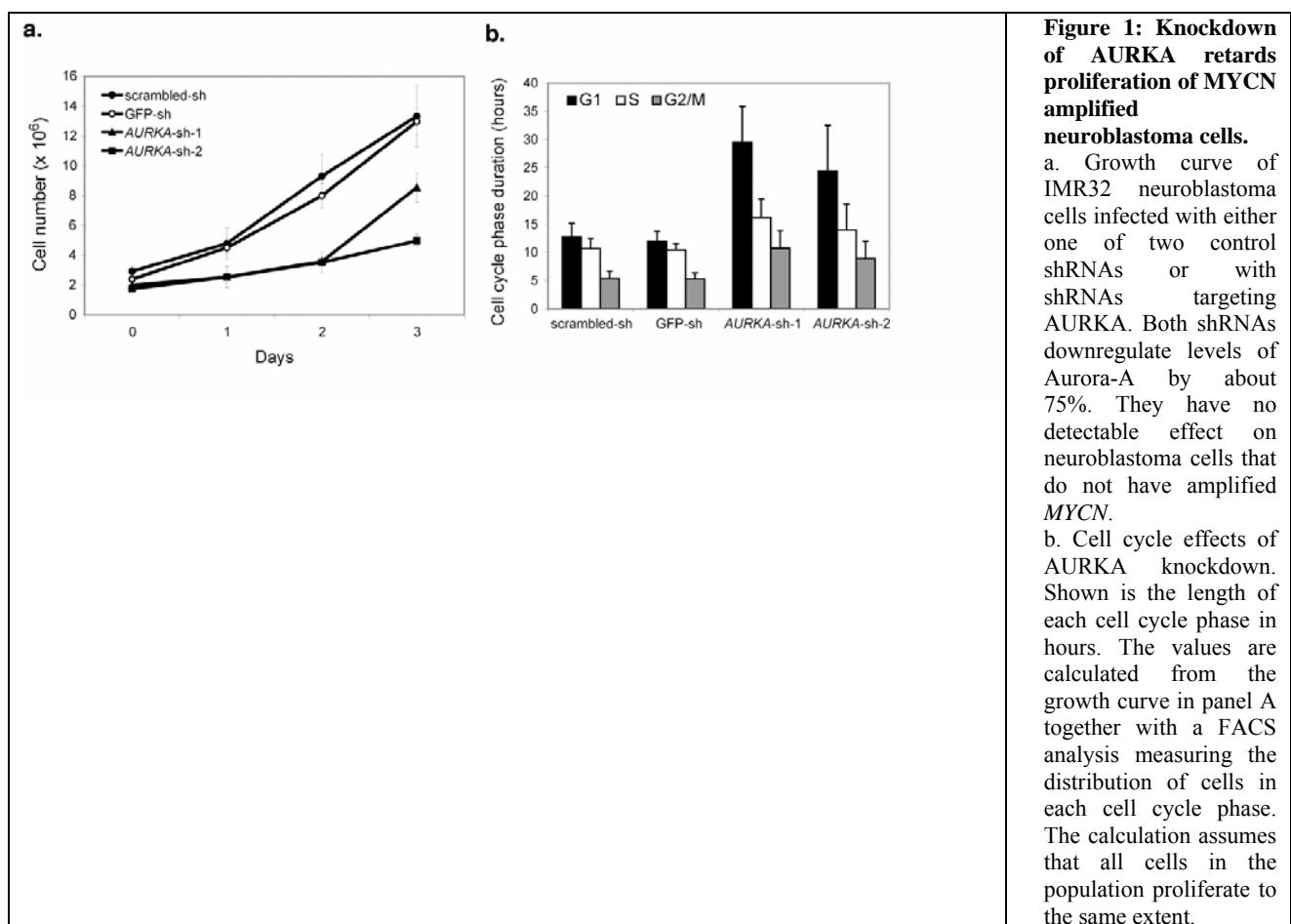
Cluster 5: WP 22

Identification of genes that show synthetic lethality with deregulation of the Myc/Miz pathway (UNIMAR)

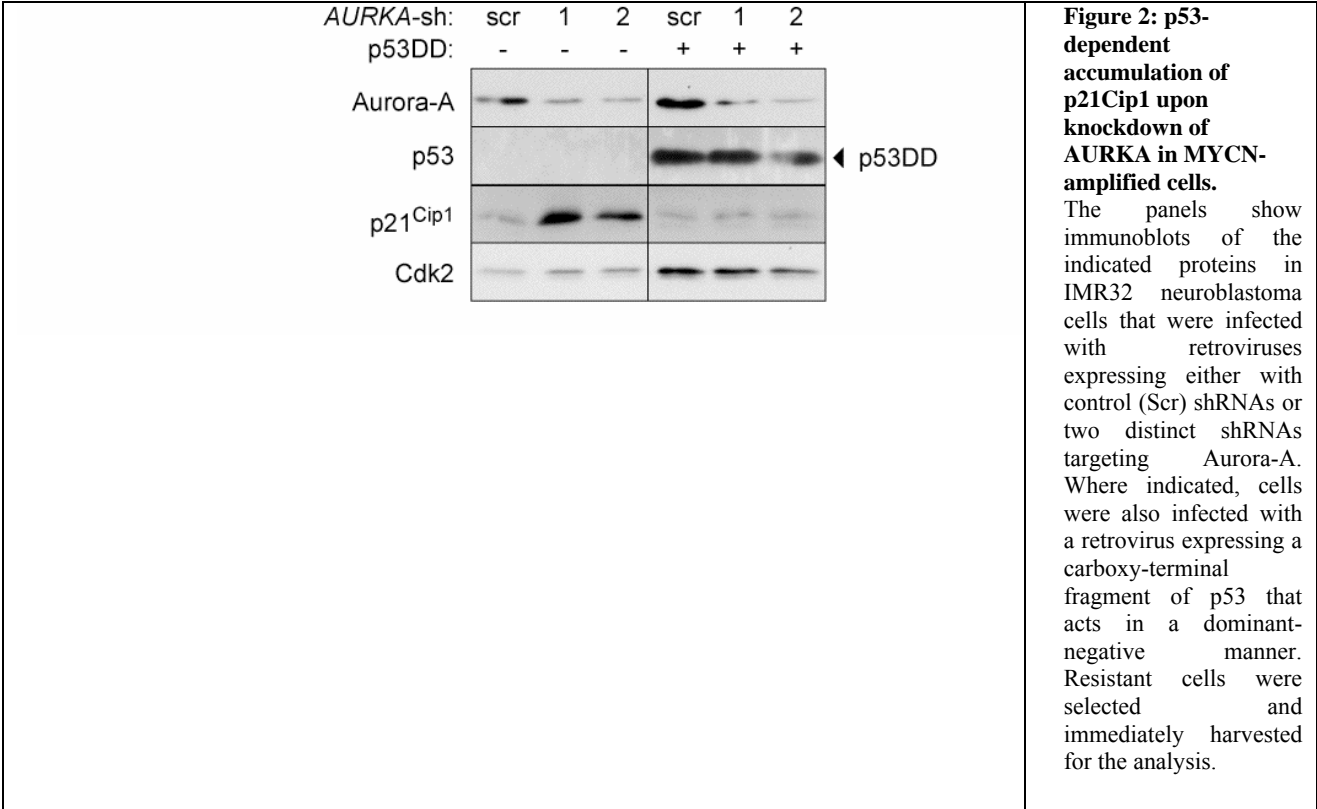
As detailed in our last report, we focused on neuroblastoma for these experiments, since there is defined subgroup of tumors that carries an amplified *MYCN* gene. We took a candidate approach and screened 600 shRNA vectors directed 200 genes that are either contained in a profile of genes that are specifically expressed in neuroblastoma cells that carry amplified *MYCN* or are known, direct target genes of Myc (the latter group contains all direct, induced target genes of Myc that were contained in a database on the day we started the experiment). We screened these genes in a pair of neuroblastoma cell lines, one of which contains amplified *MYCN* and depends on *MYCN* for proliferation, whereas the other cell line has a single copy gene that is not expressed.

From our screen, we identified 10 shRNAs that had a reproducible and differential effect. We focused in the subsequent experiments on an shRNA directed against *AURKA* (Aurora-kinase A) and have now tried to understand why *AURKA* acts in such a specific manner. These experiments are still under way.

1. Multiple shRNAs directed against *AURKA* show a clear correlation of phenotype with the knockdown efficiency, strongly suggesting that the selective suppression of colony formation in *MYCN*-amplified cells is not an off-target effect.
2. shRNAs targeting *AURKA* do not induce apoptosis, but cause a delay in cell proliferation in *MYCN* amplified neuroblastomas; they have no effect on proliferation of neuroblastoma cells lacking *MYCN* amplification. This delay occurs in all phases of the cell cycle, including the G1 phase. Therefore, the phenotype does not reflect a specific mitotic function of *AURKA* (Figure 1).



3. These effects can be partially reconstituted in cells that do not express amplified *MYCN*, but are engineered to express an NMyC-ER fusion protein; in these cells, depletion of AURKA induces a hormone-dependent delay in all phases of the cell cycle.
4. Microarray analysis identifies a class of genes that is selectively regulated by Aurora-A in *MYCN*-amplified cells, but not in control cells. This class of genes includes known target genes of p53, such as *p21CIP1* and *PLK2*. Indeed, knockdown of *AURKA* induces the selective accumulation of p53 in *MYCN* amplified cells, but has no effect in single copy tumor cells. p53 protein that accumulates in these cells is phosphorylated at serine 15, suggesting that it is active. It should be noted that neuroblastomas usually are wildtype for p53; in addition, several previous reports had suggested that Aurora-A is capable of phosphorylating and inhibiting p53. Accumulation of p53 and p21 upon knockdown of *AURKA* are also seen in NMYCER cells in a hormone-dependent manner.
5. Expression of a dominant-negative allele of p53 abolishes induction of p21Cip1 upon knockdown of *AURKA* in *MYCN*-amplified cells, arguing that p53 is indeed the critical target for Aurora-A action in this pathway (Figure 2). However, this does not rescue the suppression of colony formation and the delay in cell proliferation, demonstrating that at least one additional target for Aurora-A action must exist.



6. Initial data now show that knockdown of *AURKA* in *MYCN*-amplified cells decreases levels of N-Myc protein to a significant degree; indeed, the 3'-UTR of the *MYCN* mRNA contains a potential cytoplasmic polyadenylation element, which is known to inhibit translation via CPEB; binding of CPEB is regulated by phosphorylation by Aurora-A. It is therefore possible, that Aurora-A has two functions in *MYCN*-amplified neuroblastoma cells: enhancement of translation of the *MYCN* mRNA and restriction of p53 function. Since N-Myc enhances Aurora-A expression, this may constitute a positive feedback loop. However, more work is required to establish this latter part of the model.

In addition to these experiments, we have acquired a PATHWAY automated confocal microscope and have set up 96-well assays to screen directly for synthetic lethal interactions in a 96well format. In these assays, we initially tested a U2OS-cell line that carries a MycER fusion protein (as in WP21). When assayed at 10% FCS, these cells do not undergo apoptosis. We are now looking for shRNA vectors that have no effect in the absence of 4-OHT, but that induce apoptosis in the presence of 4-OHT. To set up the assay, we have reproduced data that the TRAIL ligand acts in such a manner. As an initial step for a screen, we have prepared all vectors targeting kinases from the NKI library and set up the required infection and analysis protocols. We believe that the initial screen of the kinase library can still be completed within the framework of this program.

Accumulative list of tasks, deliverables and milestones in the WP 22 (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T22.1	Isolate RNA and cDNA from infection of U2OS-MycER cells with entire NKI library	Achieved	M 22.1	21
	T22.2	Perform barcode analysis in Amsterdam together with NKI	Achieved	M 22.1	21
	T22.3	Characterize positive shRNAs ("Outliers") from barcode screens and perform functional tests	Achieved	M 22.2	30
	T22.4	Repeat Task 22.1 with mouse library and MEFs that express MycER.	delayed until sequence validated library is available	M 22.4	30
	T22.5	The identified genes will be compared with the genes obtained in the screens in the other workpackages, in particular from WP	Achieved (and still ongoing)	M 22.3	30
	T22.6	The expression of the identified genes in primary tumours will be analysed by database analysis and screening of multiple tumour arrays by ISH. Functional tests of positiveFunctional tests of positive inserts. Test of control shRNAs from Miz1S428A screen.	Achieved (and still ongoing)	M 22.3	30
	T22.7	Gene copy numbers for selected overexpressed or non-expressed genes will be determined by FISH	In progress	M 22.3	30
Update 12-30	T 22.8	Perform candidate screen in neuroblastoma cells	Achieved	M 22.6	24
	T22.9	Biochemical Characterization of AURKA effect in MYCN neuroblastoma cells	In progress	M 22.7	36
	T 22.10	Develop novel strategies for synthetic lethal screens in 96well format	Completed	M 22.8	48
Update 25-42	T 22.11	Screen shRNA kinase library in U20S-MycEr cells	In progress	M22.9	48

Cluster 5: WP 23

Identification of genes that promote long-term self-renewal of hematopoietic stem cells carrying oncogenic mutations in the ARF, Myc or PI3-kinase pathways (ISREC1)

The aims of this WP are to study the role of the PI3-kinase pathway (by studying Pten deficient mice/cells) during normal and malignant hematopoiesis, the presence of a possible cooperative effect between c-Myc and Pten during leukemogenesis, and to identify putative novel collaborators in this process by the use of an shRNA screen.

During 2006 we have focused first on the establishment of a novel lentiviral system that allows the inducible overexpression and/or shRNA-mediated knock-down of any gene of interest in hematopoietic stem cells (HSCs) *in vitro* and *in vivo*, as well as in leukemic cells. Such a system is crucial to validate the role of any gene identified using genetic screens *in vitro* or *in vivo*. The system contains two components: First, an inducible lentiviral vector harbouring the cDNA or shRNA of interest, and second, a transgenic mouse line engineered to ubiquitously express (including HSCs) the tTR-KRAB repressor (inhibiting both polymerase II- and polymerase III-mediated transcription) which is active in the absence, but not in the presence of doxycycline. HSCs isolated from these tTR-KRAB mice are then infected with a novel self-inactivating lentiviral vector (IKO) into which a tetO-H1 promoter-shRNA cassette has been inserted into the U3 region of the 3'LTR. In addition, the IKO vector also allows easy insertion of a cDNA that is then expressed under the control of the EF1 α or ubiquitin promoter, both highly expressed in HSCs. The presence of tTR-KRAB binding sites in the vector make both cDNA and shRNA expression dependent on the presence of doxycycline.

For the conditional expression of cDNAs or shRNAs, HSCs from tTR-KRAB mice are infected with the IKO vector. Due to the presence of tTR-KRAB, the cDNA or shRNA is not expressed. The transduced cells can then be transplanted into lethally irradiated mice, and after their engraftment (8 weeks), the mice can be treated with doxycycline to induce expression of the inserted cDNA and/or shRNA. Preliminary results show that our transgenic mice express the tTR-KRAB protein in both ear fibroblasts and HSCs, and that expression of a GFP reporter gene can be controlled in a doxycycline dependent manner (Fig. 1). Moreover, we have demonstrated that our vector efficiently transduces purified LSK-HSCs *in vitro*, and that these HSCs retain their stem cell activity *in vivo* after transplantation into competent recipients. In conclusion, our strategy permits robust and regulatable cDNA or shRNA expression, and is therefore suitable for rapid, functional *in vivo* testing of candidate genes (gain- and loss- of function) in HSCs and other hematopoietic cell types, as well as in hematopoietic malignancies.

Using a different strategy, we have characterized the effects of loss of the tumor suppressor Pten in both the hematopoietic system and during leukemia formation. To this end, we have generated a mouse model in which the *Pten* gene can be deleted in hematopoietic cells, including HSCs. This was achieved by crossing the conditional *pten*^{fllox} allele with either the interferon- α inducible MxCre or the tamoxifen inducible Scl-CreER^T transgenes. This allows conversion of the *pten*^{fllox} allele into a *pten*^Δ null allele in HSCs and other hematopoietic cell types. As a result Pten mutant mice developed massive splenomegaly accompanied by myeloid proliferative disease. Interestingly, in the bone marrow the number of phenotypic and functional repopulating HSCs progressively decreases over time (Fig. 2A). This result seems to support reports by others suggesting that loss-of PTEN causes loss of HSCs due to their increased proliferation, often associated with their subsequent differentiation and loss of self-renewal activity. However, unexpectedly, extensive cell cycle analysis using short term BrdU labeling, expression of Ki67 and label retaining assays did not reveal any differences in proliferation or quiescence between control and Pten deficient HSCs. Our data strongly suggest that Pten, and therefore the PI3-kinase pathway has little or no effect on HSC dormancy or HSC self-

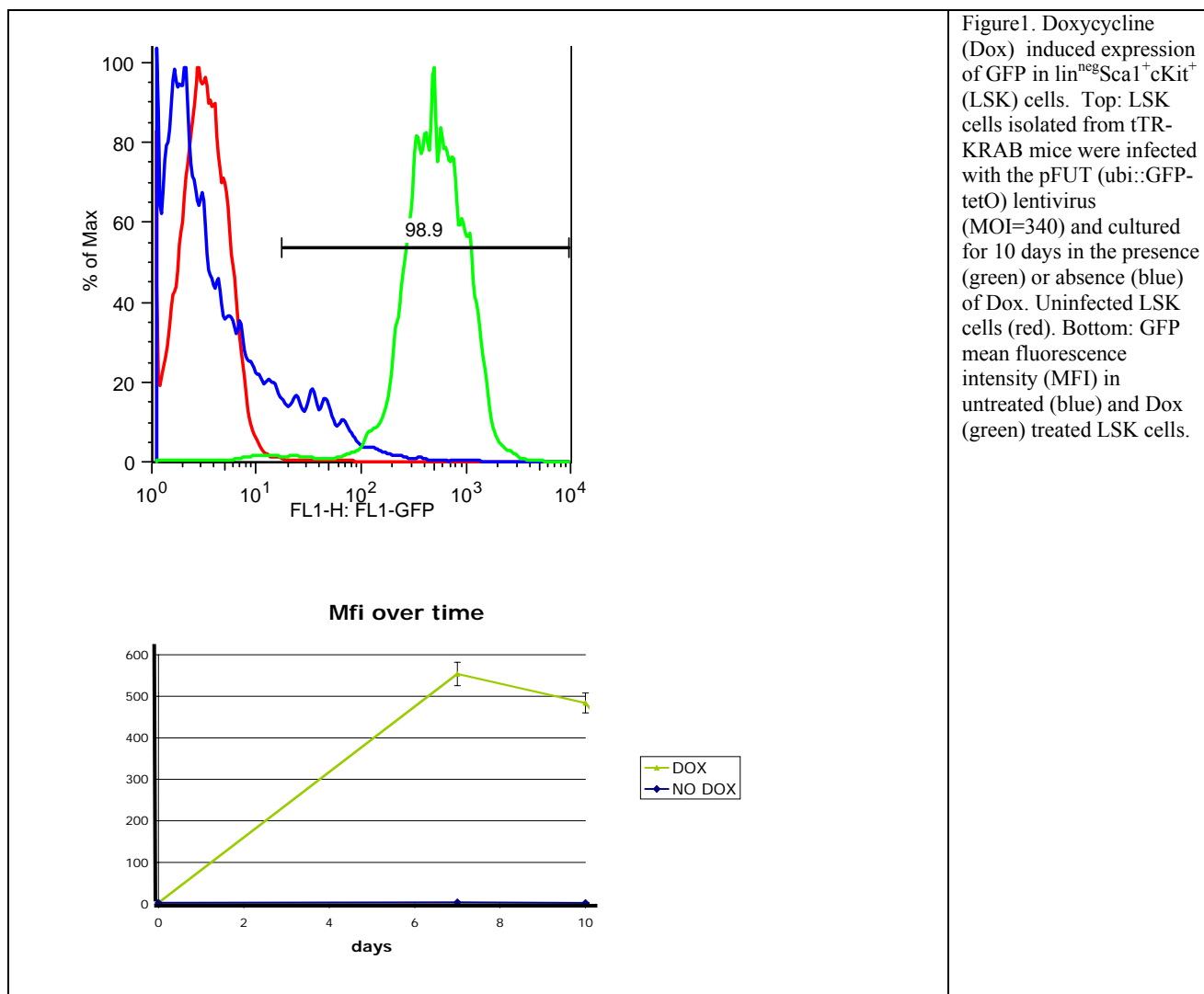


Figure1. Doxycycline (Dox) induced expression of GFP in $\text{lin}^{\text{neg}}\text{Sca1}^+\text{cKit}^+$ (LSK) cells. Top: LSK cells isolated from tTR-KRAB mice were infected with the pFUT (ubi::GFP-tetO) lentivirus (MOI=340) and cultured for 10 days in the presence (green) or absence (blue) of Dox. Uninfected LSK cells (red). Bottom: GFP mean fluorescence intensity (MFI) in untreated (blue) and Dox (green) treated LSK cells.

renewal. In order to provide an alternative hypothesis to explain the observed loss of HSCs in the bone marrow, we tested whether HSCs might have been mobilized to the spleen. Indeed, the number of phenotypic HSCs ($\text{CD34}^{\text{neg}}\text{lin}^{\text{neg}}\text{Sca1}^+\text{cKit}^+$) in the spleen increases in a similar proportion to their decrease in the bone marrow, suggesting the presence of extramedullary hematopoiesis. As a mechanism that could explain this finding, we detected dramatically increased levels of the mobilizing cytokine G-CSF in the blood serum suggesting that loss-of Pten stimulates mobilization and migration of HSC from the BM to the spleen via G-CSF. If this is indeed the case, this surprisingly indicates that the PI3-kinase pathway has no effect on HSC self-renewal (G.Oser and A. Trumpp, in preparation).

Next we assessed the tumor suppressive effects of Pten by transplanting Pten deficient BM cells into immuno-compromised mice and show the rapid development of transplantable CD4^+ T cell leukemias in its absence. Next we transplanted various hematopoietic sub-populations using c-Kit, Sca-1, CD25 and CD127 as markers to test whether only a specific population, containing a putative leukemic stem cell, is able to transplant the disease. Unfortunately, most of the transplanted sub-populations re-established the leukemia in immuno-compromised recipients with similar latency indicating that Pten-deficient CD4^+ T cell leukemias may not be driven by leukemic stem cells.

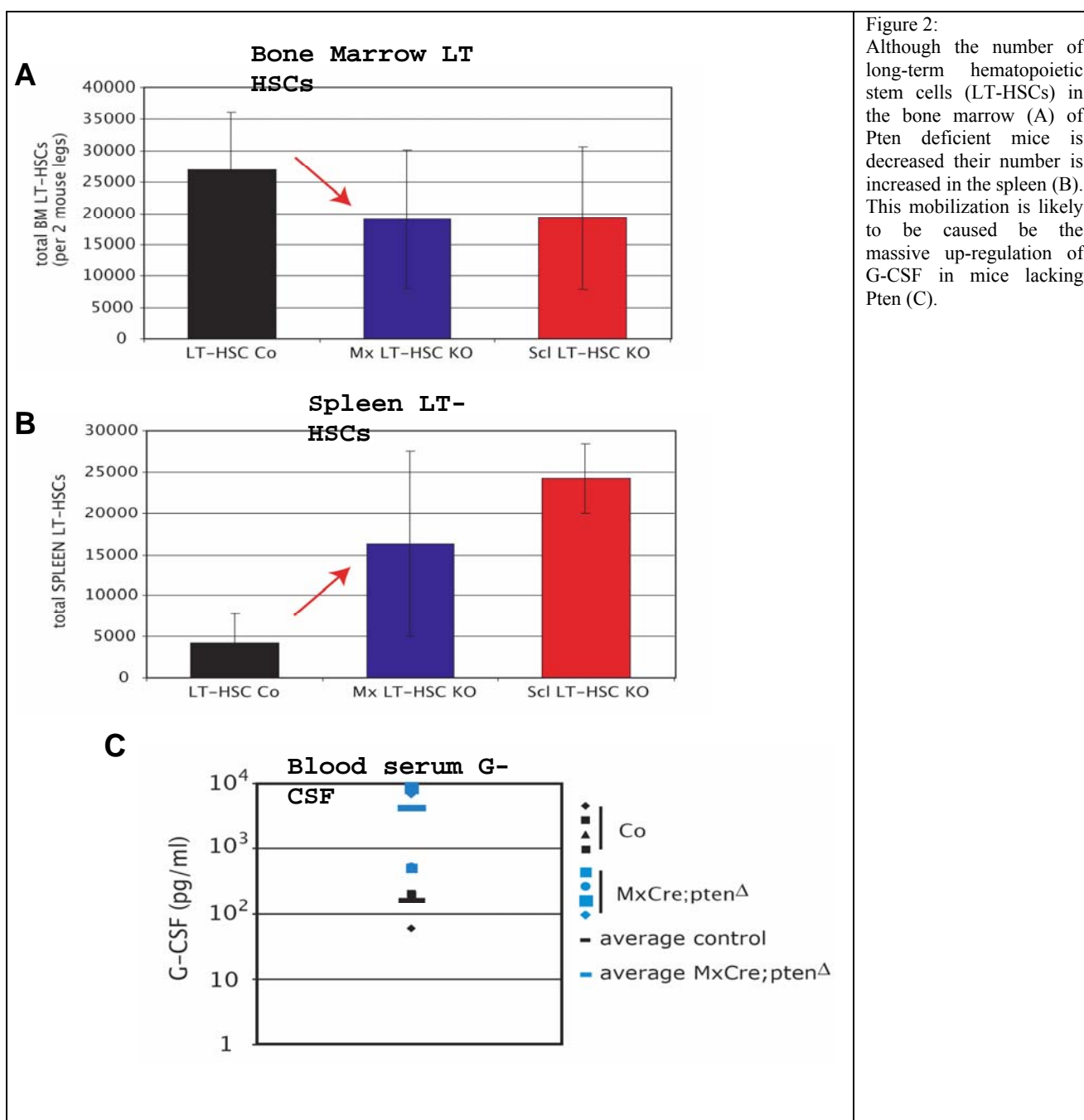


Figure 2: Although the number of long-term hematopoietic stem cells (LT-HSCs) in the bone marrow (A) of Pten deficient mice is decreased their number is increased in the spleen (B). This mobilization is likely to be caused by the massive up-regulation of G-CSF in mice lacking Pten (C).

To address a possible cooperation between Pten and the oncogene c-Myc, we infected Pten-deficient fetal liver cells with a retrovirus expressing human c-MYC, and transplanted them into lethally irradiated recipients. The two oldest mice, currently 2 months post transplant, show between 5% and 20% chimerism for the c-MYC vector but appear healthy and so far show no obvious signs of tumor development. We expect them and others in the cohort to soon develop tumors. In an alternative approach, we have generated SCL-tTA;TRE-MYC; MxCre-PTEN^{flx/flx} mice which will allow us to simultaneously induce c-Myc expression and deletion of the Pten gene in adult HSCs.

One of the goals in this WP is to identify novel collaborators of c-Myc and Pten during HSC and leukemic stem cell function using shRNA screens. Since the availability of the MOUSE shRNA library was still uncertain in the first half of 2006 we mainly focused on the projects described above, but will commence

Third Annual Periodic Activity Report (2006)

with these experiments in 2007. In a side project we have shown that although c-Myc is dispensable for normal skin homeostasis, c-Myc deficient epithelium is resistant to activated Ras mediated tumorigenesis. This is due to the ability of c-Myc to repress the CDK inhibitor p21^{Cip1}, since c-Myc /p21^{CIP1} double deficient skin re-acquires sensitivity to Ras mediated skin tumorigenesis (Oskarsson et al., (2006) Genes Dev 20: 2024-2029).

In summary, we have shown a novel role for Pten/PI3-kinase during normal and malignant hematopoiesis and have generated a novel, inducible lentiviral system to study the role of any gene of interest during hematopoietic development, homeostasis and disease.

Objectives (revised due to change of strategy)

- Identification and characterization of HSCs lacking Pten *in vivo* and *in vitro*
- Identification of genes which promote self-renewal of c-Myc⁺ Pten^{Δ/Δ} HSCs
- Generation of a novel lentiviral system to inducibly express any cDNA or shRNA *in vitro* and *in vivo*.

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T23.1	Establishment of LTC-IC assays	Achieved	M 23.1	18
	T23.2	Establishment of viral vectors that can be used to overexpress c-MYC	Achieved	M 23.2	21
	T23.3	Production of high titer viruses to infect HSCs	Achieved	M 23.2	21
	T23.4	Isolation of <i>PTEN</i> ^{Δ/Δ} hematopoietic stem cells (HSCs)	Achieved	M 23.3	30
Update 12-30	T23.5	Isolation of HSCs with the following genotypes: <i>p19ARF</i> ^{-/-} ; <i>PTEN</i> ^{Δ/Δ} , and <i>p19ARF</i> ^{-/-} ; <i>PTEN</i> ^{Δ/Δ} doubles.	Cancelled	M 23.3	30
	T23.6	Generation of HSCs which overexpress c-Myc	Achieved	M 23.3	30
	T23.7	Generation of HSCs which overexpress c-Myc on a <i>PTEN</i> ^{Δ/Δ} background	In progress	M 23.3	30
	T23.8	Generation of HSCs which overexpress c-Myc on a <i>p19ARF</i> ^{-/-} background	Cancelled	M 23.3	30
	T23.9	Introduction of the SCA-1::GFP allele into the different mutant backgrounds	Cancelled, alternative strategy	M 23.4	30
	T 23.10	Determination of the time point by which each genotype has lost HSC activity in vitro	Achieved and in progress	M 23.3	30
Update 25-42	T23.11	Determination of culture time c-Myc ⁺ Pten ^{Δ/Δ} HSCs are lost	Achieved	M 23.5	48
	T23.12	Carry out shRNA screen for vectors that promote c-Myc ⁺ Pten ^{Δ/Δ} HSC self-renewal or survival	Not started yet	M 23.5	48
	T23.13	Functional tests of positive inserts	Not started yet	M 23.5	48
	T23.14	Sort different leukemic sub-populations and transplantation into NOD/SCID mice.	achieved	M 23.6	48
	T23.15	Microarray analysis of purified leukemic stem cells	Cancelled, alternative strategy	M 23.6	48
	T23.16	Establishment of an inducible lentiviral system to overexpress or knock down genes in hematopoietic stem cells in vitro and in vivo	Achieved	M 23.7	48
	T23.17	Establish the role of Pten in normal and malignant hematopoiesis	Achieved	M23.8	36

SUMMARY of research activities during 2006**Cluster 6 (WPs 24-26): Identification of new targets by synthetic lethality screens of preexisting tumor models (NKI2, MDC (coordinator), NKI1)**

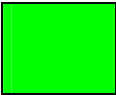
The principal objective of cluster 6 is the identification of new targets via synthetic lethality and related genetic screens in *in vitro* and *in vivo* tumor models. So far, cluster 6 has accomplished to set up various models to screen for genes that interfere with drug-mediated death or arrest in either unbiased or predetermined genetic scenarios such as p53 deficiency. Specifically, cluster 6 employed both cell culture and mouse models as platforms to retrieve genetic hits from genome-wide screens and to validate candidate genes in functional assays *in vitro* and *in vivo*. ShRNA-based screens have identified genes that confer resistance to experimental anticancer agents such as the HDAC inhibitor SAHA and Nutlin, a p53-(re-)activating compound targeting the Mdm2/p53 interaction. Retroviral screens based on insertional mutagenesis, and, ongoing experiments based on different genome-wide mouse siRNA libraries as well as educated pathway guesses have led to numerous candidate loci that may compromise drug-inducible senescence in aggressive, apoptosis-deficient lymphomas. Moreover, monitoring tumor development, progression and regression in mice engineered to harbor conditional genetic lesions by bioluminescence imaging is now an established platform that will be utilized in further complex functional analyses to identify and validate genes involved in anticancer drug responses. Accordingly, work during the next 12 months will focus on the following two prime objectives: (i) identification and validation of genes that compromise critical aspects of anticancer drug action such as apoptosis and cellular senescence, including synthetic lethality screens; (ii) functional imaging of anticancer drug action dependent on the presence or absence of candidate lesions in tractable mouse tumor models *in vivo*.

Goal	Partner	Work planned for the next 18 months							
		Set-up of screening		Genome-wide screen		Validation of hits		Functional data	
To perform bar coded RNAi and candidate synthetic lethality screens with anticancer drugs	NKI-2								
To identify and validate genes that modulate sensitivity to clinically relevant drugs	NKI-2								
To identify genes that confer escape from drug-inducible senescence in aggressive B cell lymphomas in vitro and in vivo	MDC								

Third Annual Periodic Activity Report (2006)

To monitor drug responses using fluorescent reporters in live mice over time	MDC								
To generate bioluminescence reporter mice for in vivo pathway evaluation	NKI-1								
To determine the effect of candidate pathway defects and screening-derived lesions on chemosensitivity in conditional tumor models in vitro and in vivo	NKI-1								

Work completed during months 1-36:



Work to be done during months 37-48:



Cluster 6: WP 24

Bar coded RNAi screens to identify synthetic lethal interactions in mammalian cells (NKI2)

The goal of this workpackage is to identify genes whose inactivation alters the sensitivity of cancer cells to known anti cancer drugs. To accomplish this, we infect human tumour cell lines with the RNA knockdown library, and expose cells to anti-cancer drugs that are used in the clinic. Cells harboring the knockdown vector set are exposed to various concentrations of drug and the abundance of knockdown vectors is determined relative to a control cell population that was not exposed to drug, using the bar code screening approach. This allows us to identify genes whose suppression enhances the toxicity of known anti-cancer drugs.

In the first year, we have used shRNA bar code screens to identify genes that confer resistance to two experimental anti-cancer drugs: Nutlin and SAHA. The manuscript describing the Nutlin screen was published in 2006.

In year two, we have initiated shRNA bar code screens to identify genes that modulate cellular responses to two other cancer drugs: herceptin and Iressa. Herceptin targets the HER2/NEU protein in breast cancer. Iressa is a small molecule inhibitor of the EGF receptor. We have for each drug identified suitable cell lines and determined the minimum drug concentration to prevent colony outgrowth in the presence of drug. The Herceptin experiment identified knockdown of the tumor suppressor PTEN as a modulator of sensitivity to Herceptin. Since PTEN acts in the PIK3CA pathway and since PIK3CA is frequently mutated in breast cancer, we are currently sequencing the PIK3CA gene in breast cancer patients of known response to Herceptin. This will tell us if mutations in PIK3CA are responsible for Herceptin non-responsiveness in the clinic.

While the original goal of WP24 was to identify “synthetic lethal” interactions of cancer drugs, we have become to appreciate that the main problem with many cancer drugs is (acquired) resistance to the drugs. We therefore have shifted the focus in this WP towards the identification of genes whose inactivation confers resistance to cancer drugs. Such genes may serve as biomarkers to identify patients that fail to respond to cancer drugs. We have therefore amended the goal of this WP to identify genes that “modulate” sensitivity to cancer drugs, rather than focus on the synthetic lethal interactions only.

For the screen for resistance to Iressa (Gefitinib), we have used H-1650 Non small cell lung cancer cells, as these cells have a mutant EGFR and are very sensitive to Iressa. The outline of the screen is shown in Fig. 1 (left). To date, this screen has yielded a series of some 39 candidate genes that are potentially involved in Iressa resistance in lung cancer (Fig. 1).

In a related screen, we have identified genes whose inactivation renders cells resistant to retinoic acid induced differentiation. We used mouse F9 teratocarcinoma cells for this screen and the mouse shRNA library generated in workpackage 2. To date, this screen has identified two genes whose inactivation causes resistance to Retinoic Acid (RA). Both genes have been validated with multiple independent shRNAs, effectively ruling out that so called “off target” effects cause the phenotype. One of the hits is the heterodimerization partner of RAR-alpha, RXR. This validates the screen as RAR requires RXR to mediate differentiation. The further analysis of the second hit in the screen, a zinc finger protein, will be pursued in the final year of the project.

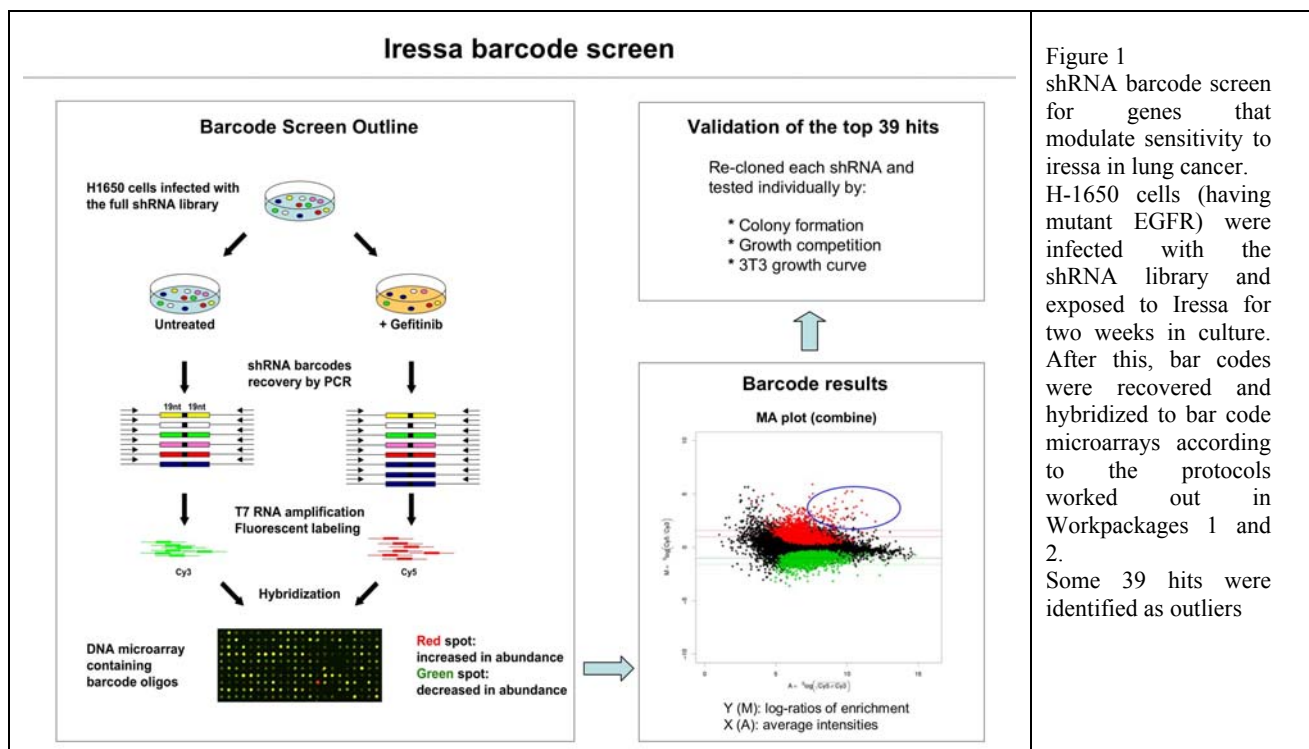


Figure 1
shRNA barcode screen for genes that modulate sensitivity to Iressa in lung cancer. H-1650 cells (having mutant EGFR) were infected with the shRNA library and exposed to Iressa for two weeks in culture. After this, bar codes were recovered and hybridized to bar code microarrays according to the protocols worked out in Workpackages 1 and 2. Some 39 hits were identified as outliers

Accumulative list of tasks, deliverables and milestones in the WP (months 1-42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 24.1	Perform bar coded RNAi screens with known anti cancer drugs	Achieved	M 24.1	24
	T 24.2	Identify candidate synthetic lethal interactions with cancer drugs	Partially achieved	M 24.2	24
Update 12-30	T 24.3	Identification of genes that modulate sensitivity to Herceptin	achieved	M24.3	36
	T 24.4	Identification of genes that modulate sensitivity to Iressa	In progress	M24.4	36
Update 25-42	T 24.5	Validation of identified gene-drug interactions	In progress	M 24.5	42
	T 24.6	Study of molecular basis of identified gene-drug interaction	In progress	M 24.6	48

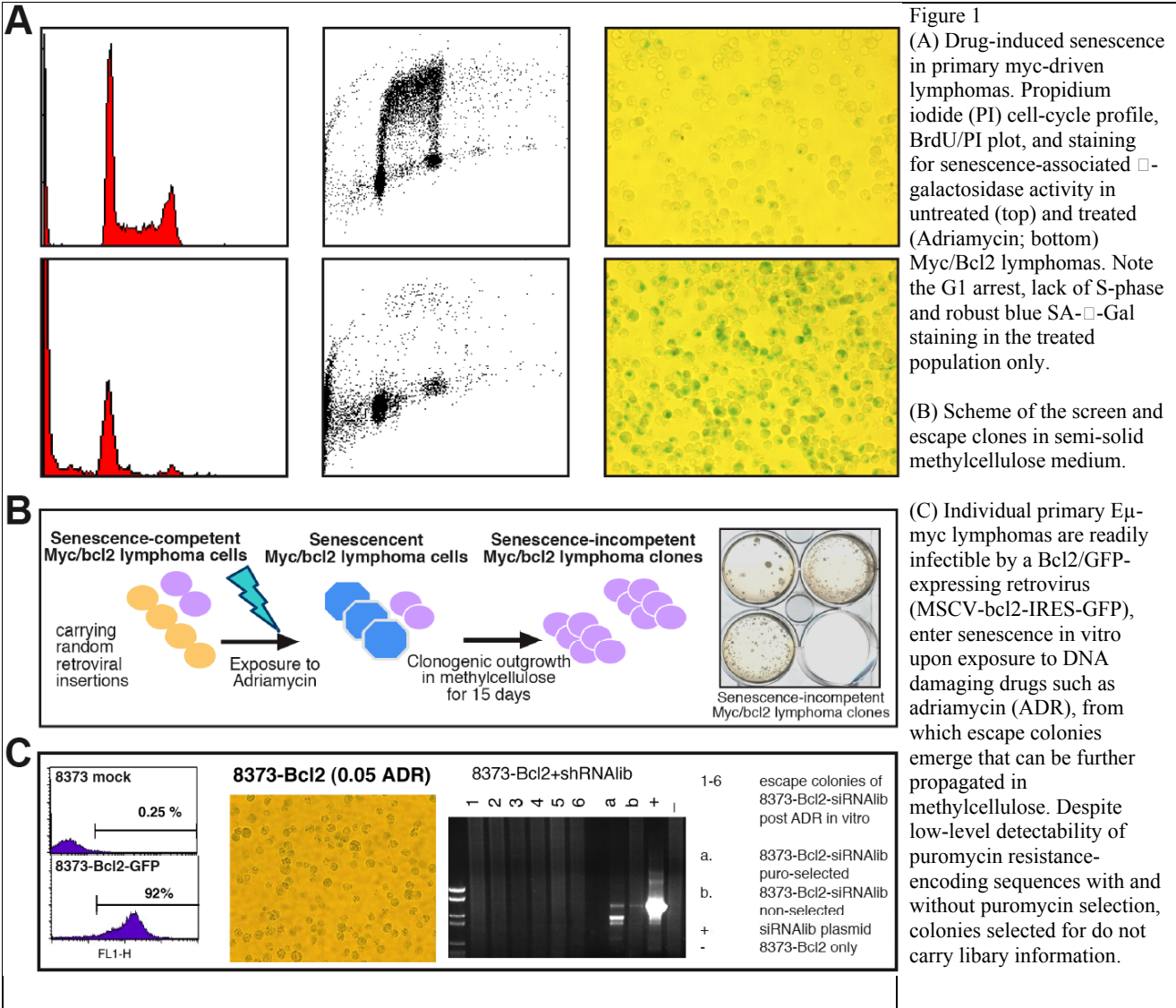
Cluster 6: WP 25

Screening for drug resistance in primary lymphomas *in vitro* and *in vivo* (MDC)

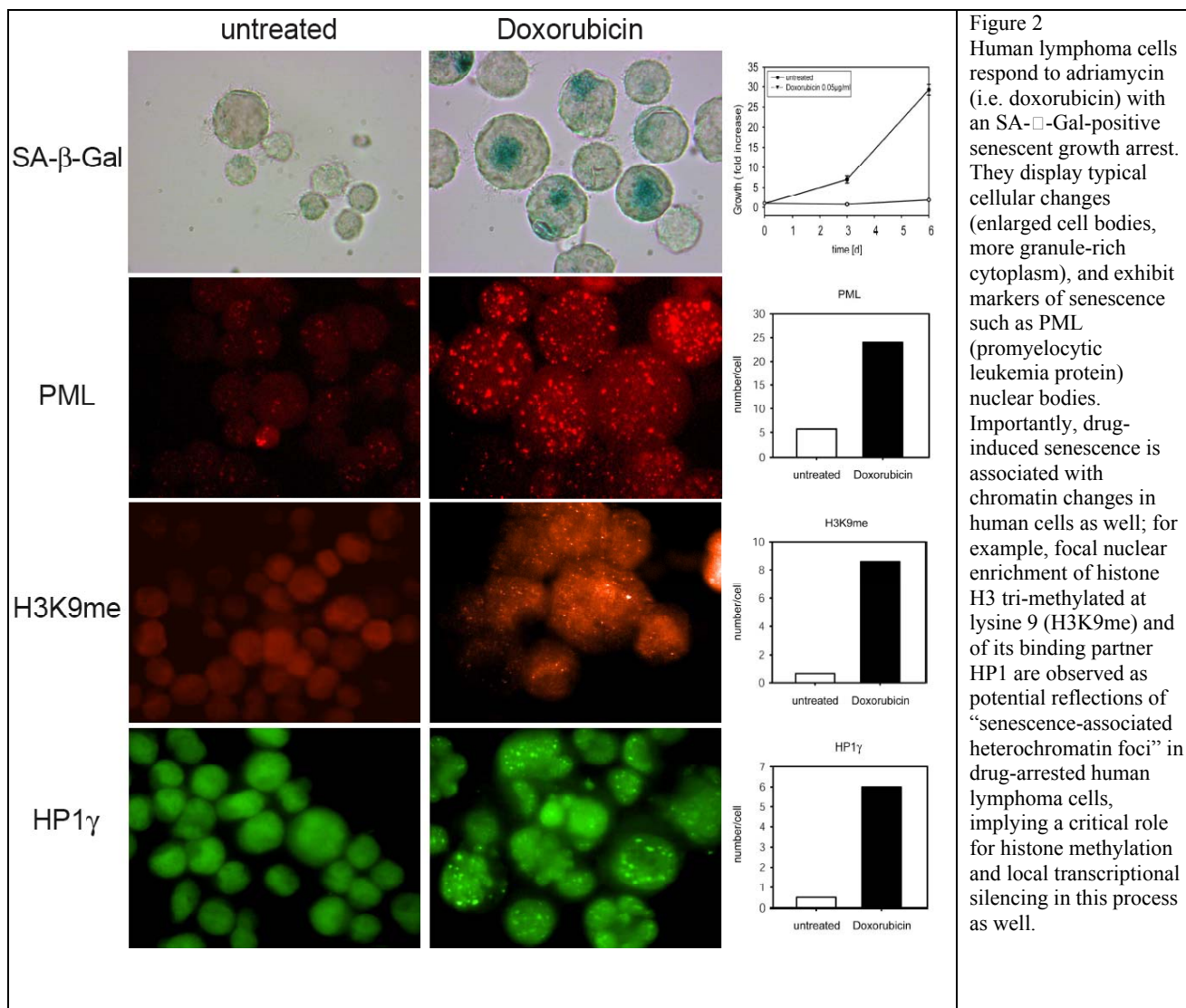
DNA damaging anticancer agents exert their therapeutic potential not only by triggering apoptosis. Particularly when apoptosis is blocked, these compounds may provoke cellular senescence, another stress-responsive program that terminally arrests the cell in the G1 phase of the cycle. Importantly, both programs – apoptosis and cellular senescence – have been shown to contribute to the outcome of cancer therapy. It is the main objective of WP25 to identify and validate genes that confer resistance to chemotherapeutic treatment by conducting *in vitro* and *in vivo* screens in the versatile *Eμ-myc* transgenic mouse lymphoma model. In this model, lymphomas arise as a consequence of Myc activation by about three to nine months of age. Primary lymphoma cells can be isolated and expanded in culture, or may be propagated in immunocompetent recipient mice upon transplantation, where they form lymphomas at sites indistinguishable from the initial transgenic animal they were derived from. Moreover, lymphoma cells are susceptible to retroviral transduction during short-term culture, allowing us to introduce retroviral cassettes for insertional mutagenesis, specific candidate genes such as the anti-apoptotic moiety Bcl2 to force lymphomas to enter senescence instead of apoptosis upon drug therapy, or to stably introduce a retroviral siRNA library to conduct genome-wide screens. These genetically modified lymphoma cells are subsequently used to primarily screen for and to validate genes involved in the escape from drug-inducible senescence.

So far, we have successfully generated a large number of primary lymphomas that were subsequently stably transduced with the apoptotic inhibitor *bcl2* to produce senescent lymphomas in response to anticancer drug treatment. Specifically, we produced matched pairs of lymphomas that were initially able to enter cellular senescence upon exposure to anticancer agents *in vitro*, but emerged as escape colonies in semi-solid medium. Several rounds of anticancer treatment and subsequent colony formation in methylcellulose were applied to select for a particularly robust phenotype. These escapees were tested for retroviral insertions in candidate genes by ‘Splinkerette’ inverse-like PCR methodology. As a consequence of retroviral insertion into a gene or its regulatory elements, gene expression might be up- or downregulated. A first list of candidate loci in whose vicinity insertions were found are to be validated in the primary lymphoma versus the escapee clone with and without chemotherapeutic challenge. Given the technical superiority of the already initially proposed siRNA library-based screening for escapees from drug-inducible senescence, we re-directed our research towards the use of the murine genome-wide siRNA library when it became available to the consortium. Furthermore, our mouse model is set up to directly test the biological impact of candidate genes identified in other WPs of this Integrated Project on lymphoma development or treatment responses.

In 2006, we started to mass-infect Bcl2-protected lymphomas with the consortium-generated siRNA library to obtain matched pairs of pre/post-treatment lymphomas that selected for a senescence-incompetent phenotype driven by a relevant siRNA. This approach is ongoing, although intense efforts based on numerous technical modifications have led to the conclusion that repetitive infections of lymphoid cells (which are known to be particularly difficult to transduce) with optimized high-titer retroviral supernatant do not result in a sufficient representation of the siRNA library. Hence, siRNA-positive lymphoma cells can be selected for under exposure to puromycin, but represent too little complexity, or re-grow as spontaneous escapees irrespective of an siRNA integration if no puromycin selection was applied (Figure 1). In addition, we have been trying to transplant library-mass infected lymphomas that were positive for retroviral integrations into mice to conduct the proposed *in vivo* screens. However, as experienced by other groups working with similar constructs in the field, we face the problem that lymphoma cells carrying the construct are never selected for *in vivo*. We currently test the hypothesis that puromycin, unlike GFP, may account for rejection. This issue will be discussed in the consortium and has led to changes strategic changes. Therefore, we currently pursue an additional, GFP-co-encoding lentiviral approach (see below).



Moreover, based on the mechanistic insights into oncogene-provoked senescence obtained in a Ras-driven mouse model in vivo, we now explored the role of the histone methyltransferase Suv39h1 in drug-inducible senescence. Myc-driven lymphomas proficient for or lacking Suv39h1 were generated and tested for the long-term impact of this gene locus on the outcome to cancer therapy in vitro and in vivo. Indeed, absence of Suv39h1 conferred resistance to drug-inducible senescence in vitro, resulting in an inferior outcome of mice being treated with Suv39h1-deficient lymphomas in vivo. Possibly reflecting a Suv39h1-like activity, we detected in drug-senescent human lymphoma cells in addition to PML bodies a focal induction of lysine 9-methylated histone H3 accompanied by the occurrence of HP1, indicating that epigenetic modifications apply to drug-inducible senescence of human cells as well (Figure 2).



To ultimately generate RFP-based reporter mice we have tested several generations of red fluorescent proteins, including dsRed1, dsRed2, HcRed, and JRed, as reported. Unfortunately, none of these fluorescent proteins achieved long-term high-level expression in hematopoietic cells, as required for our studies, although expression in fibroblast culture systems could be demonstrated. Very recent and promising results have now been obtained by testing various forms of the so called Tomato-RFP constructs (kindly provided by Roger Tsien's lab).

Objectives (revised due to change of strategy and novel data)

- Screen for and test candidate genetic defects that mediate escape from drug-induced senescence in vitro and in vivo using retroviral insertional mutagenesis, gene siRNA transfer (including genome-wide siRNA libraries) and cDNA microarrays in myc/bcl2 transgenic B cell lymphomas
- Screen human B cell lymphomas for mutants that license escape from drug-inducible senescence as well
- Test the impact of Suv39h1 inactivation in murine B cell lymphomas exposed to anticancer therapy in vivo
- Create and utilize GFP- and RFP-coexpressing retroviral constructs for functional imaging of drug effector programs in vitro and in vivo in viable mice

Third Annual Periodic Activity Report (2006)

Accumulative list of tasks, deliverables and milestones in the WP (months 1-42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 25.1	Generation of lentiviral constructs for p53 and E2F target genes that express RFP (dsRed2) as a reporter gene	Revised	M 25.1	6
	T 25.2	Generation of Eμ-myc/Eμ-bcl2 double-transgenic primary B cell lymphomas	Achieved	M 25.3	6
	T 25.3	Monitor drug responses of primary GFP-tagged lymphomas with and without RFP reporter constructs in viable animals by fluorescence imaging	(Partially) Achieved	M25.2	6
	T 25.4	Generation of myc/bcl2 double-transgenic lymphomas that carry a definite or progressive number of proviral integration sites by retroviral insertional mutagenesis	Achieved	M25.4	12
	T 25.5	Selection of clonal lymphoma cell populations that escaped from drug-induced senescence upon multiple rounds of clonogenic assays	Achieved	M25.4	12
	T 25.6	Isolation of apoptosis-defective lymphomas that became refractory to drug-inducible senescence upon multiple administrations of chemotherapy in vivo	Achieved	M25.5	18
	T 25.7	Evaluation of differential gene expression in senescence-incompetent versus –competent cell populations derived from the same primary lymphoma by cDNA array based expression profiling	Not started yet	M25.9 and M25.10	27 and 30
	T 25.8	Identification of proviral integration sites in senescence-incompetent lymphoma cells by inverse PCR and subsequent sequencing	Achieved	M25.6 and M25.8	18 and 24
Update 12-30	T 25.9	To generate human lymphoma cell lines that can be screened for drug-inducible senescence upon infection with a human siRNA library	Achieved	M25.7	21
	T 25.10	To establish additional markers for a more complex characterization of the senescent phenotype	Achieved	M25.11	30
	T 25.11	Generation of retroviral constructs for p53 and E2F target genes that express RFP (dsRed-Tomato) as a reporter gene	In progress (but revised)	M25.12	45
	T 25.12	Monitor drug responses of primary GFP-tagged lymphomas with and without RFP reporter constructs in viable animals by fluorescence imaging	In progress	M25.12	48
Update 25-42	T 25.13	Assess the impact on the long-term outcome to cancer therapy in Suv39h1-deficient mouse lymphomas in vivo	In progress	M 25.13	42
	T 25.14	Explore a putatively drug-inducible Suv39h1/H3K9me3/HP1 senescence signature in human lymphoma cells	In progress	M 25.14	42

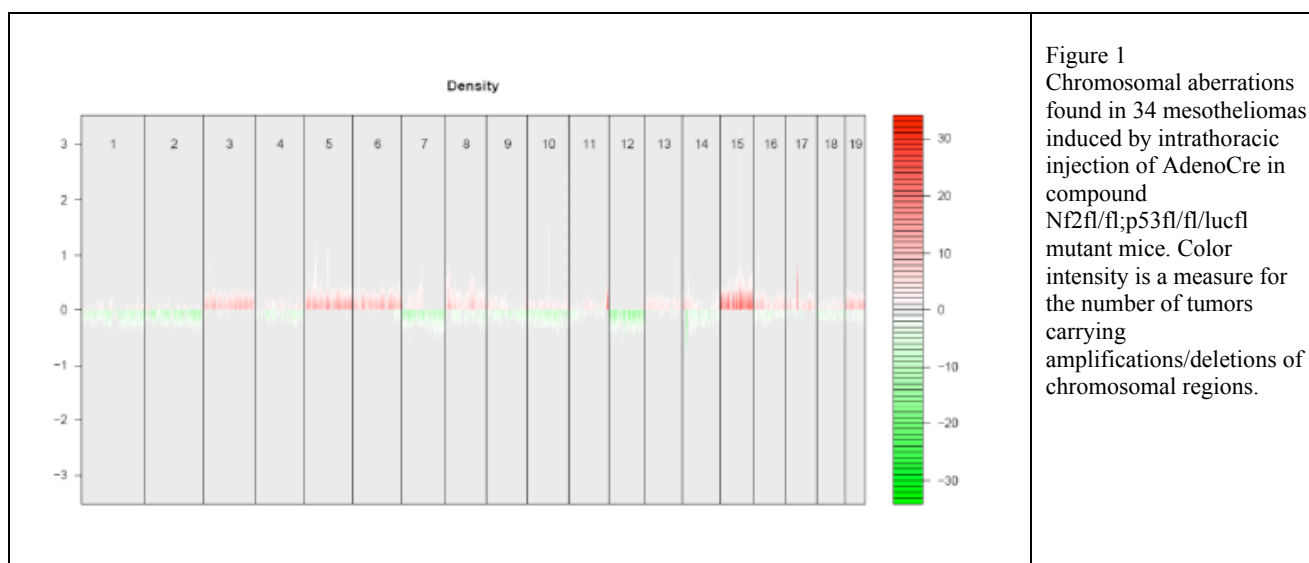
Cluster 6: WP 26

The role of distinct cancer pathways in the resistance to therapy (NK11)

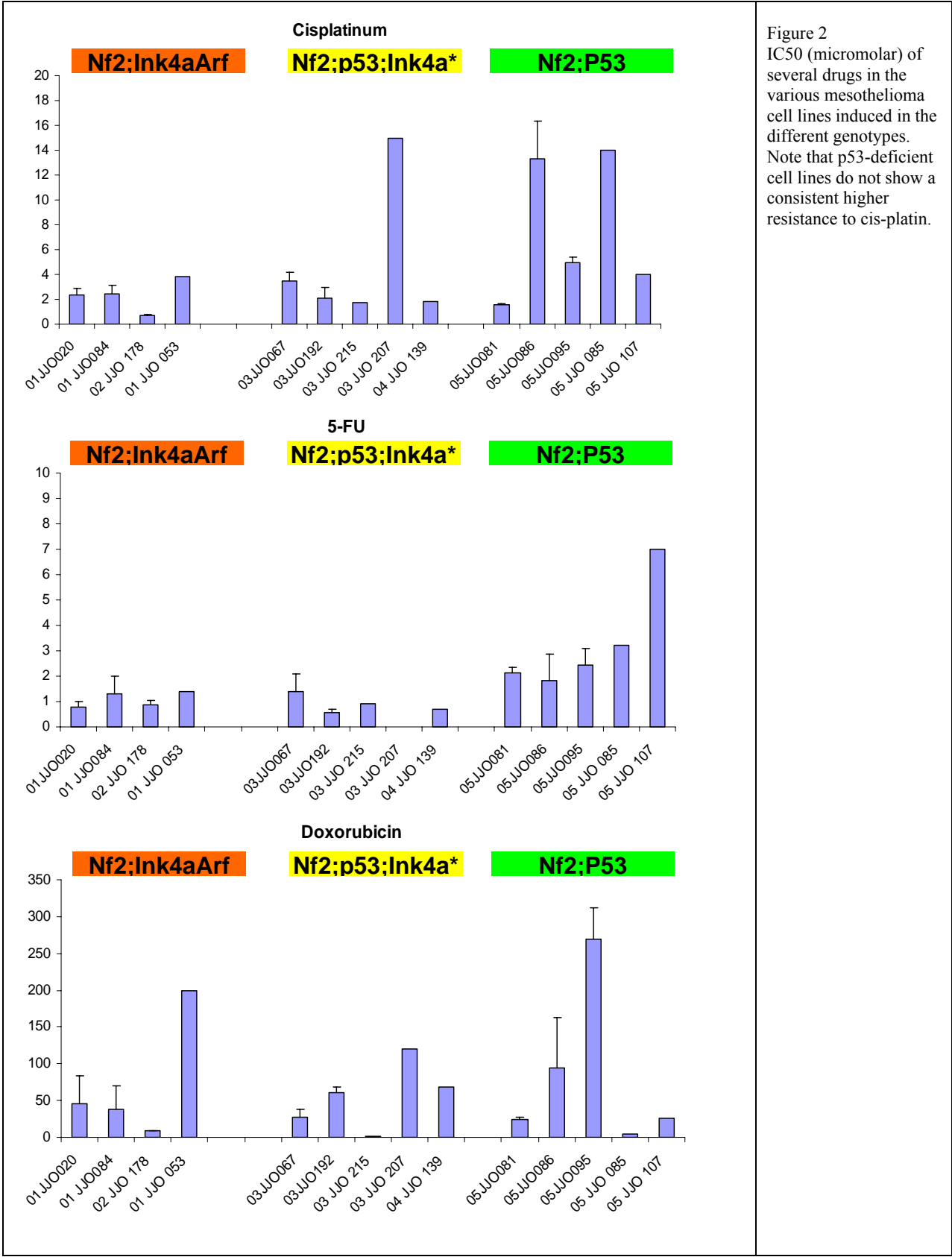
The main objective of WP26 was to determine whether loss of p53 in non-lymphoid tumor models is a major factor in enhancing chemotherapy resistance as has been observed in Emu-Myc tumor models studies by Clemens Schmitt and Scott Lowe. We have attempted to study this in what seemed a suitable model: pituitary tumors induced in Rbflox/flox and Rnflox/flox;p53flox/flox mice. However we could not well address the relevance of p53 loss in this system for chemotherapy resistance as the tumor type changed as soon as additional lesions were combined with Rb loss.

In 2006 we have after attempts were made to address the question in different backgrounds (p53 heterozygosity and Ink4aArf deficiency) abandoned the the pituitary model altogether. We have concentrated on a mesothelioma model in which we observed very similar tumors in either a Nf2flox/flox;Ink4aArf-flox/flox or a Nf2flox/flox;p53flox/flox background. We have isolated cell lines of tumors induced in the following genotypes:

Nf2-fl/fl;Ink4aArf-flox/flox;Actin-Luc-flox, Nf2-flox/flox;p53-flox/flox;Actin-Luc-flox, and Nf2-fl/fl;p53fl/fl;Inka*/*;Actin-Luc-flox. 5 cell lines were obtained from each and characterized by resistance profiles against cytotoxic drugs used for treating mesotheliomas clinically. In addition we have partly analyzed these tumors by CGH analysis and expression array profiling.



CGH analysis shows that mesotheliomas in p53 conditional models showed more chromosomal aberrations (fig 1) than mesotheliomas in which Ink4aArf was disrupted. c-Myc amplification was regularly found. c-Met amplification was identified in a small subgroup of tumors. We have determined whether orthotopically grafted tumor cell lines derived from AdenoCre induced tumors in Nf2flox/flox;p53flox/flox;Inka*/*;Lucflox mice showed sensitivity to Thalidomide. Even at the highest tolerated dose no significant effect of Thalidomide treatment was observed. Tumors grew aggressively without being delayed by the treatment.



Third Annual Periodic Activity Report (2006)

We have started to make an inventory of the sensitivity of the cell lines to cytotoxic drugs. A few examples are shown in fig. 2. The cell lines showed a substantial individual variation in drug sensitivity. We did not see a clear correlation with the p53 status. Tumors that were wildtype for p53 did not show consistent more susceptibility to cytotoxic drugs as one might have expected. It will be important to determine whether this same behavior is also observed in vivo in primary tumors. We have shown that orthotopic grafting of the cell lines closely reproduce the phenotype of the spontaneous tumors. Subsequently, the resistance profile of spontaneous and orthotopically grafted cell lines can

Objectives (revised due to change of strategy)

- Completely switch from the pituitary tumor to mesothelioma. The latter model allows the study of spontaneously occurring tumors, tumors arising after orthotopic grafting of established cell lines and primary tumor cell suspensions, and the analysis of cell lines in vitro. We will test the p53 dependence or lack thereof in the various genotype in these complementary models.

Accumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 26.1	Generation of versatile in vivo bioluminescence reporter mice to study tumour growth and tumour regression in response to chemotherapy or specific pathway inhibitors to be utilized in 2 nd period of the program	Achieved	M 26.1	6
	T 26.2	Determine effect of p53 loss on regression of pituitary tumours in Rbflox/flox;POMC-Cre;POMC-luciferase compound mutant mice in response to doxorubicin with or without a CDK2 inhibitor.	Cancelled	M 26. 2	21
	T 26.3	Determine effect of Ink4a/p19Arf loss on regression of pituitary tumours in POMC-Rbflox/flox;POMC-Cre;POMC-luciferase compound mutant mice in response to doxorubicin with or without a CDK2 inhibitor	Abandoned	M 26. 3	24
Update 12-30	T 26.4	Derive cell lines from mesothelioma's induced in the various mesotheliomas mouse models.	Achieved	M 26. 4	21
	T 26.5	Compare growth characteristics and chemotherapy resistance of Nf2;p53 and Nf2;Ink4a/p19Arf mutant cell lines	In progress	M 26. 5	30
	T 26.6	Perform CGH and expression array analysis to search for new targets for intervention.	In progress	M 26. 6	30
	T 26.7	Show effects of specific inhibition of genes found in task 6 on growth of cells in vitro and in vivo.	Not started yet	M 26. 7	33
Update 25-42	T 26.8	Show specificity of siRNA against tumor cells but not normal cells	Not started yet	M 26.8	42
	T 26.9	Show inhibitory effect of inducible siRNA (if available) in cell graft models	Not started yet	M 26.9	45
	T 26.10	Show inhibitory effect of inducible siRNA (if available) in spontaneous tumor model	Not started yet	M 26.10	48
	T26.11	Explore effectiveness of inhibitors of vascularization on mesothelioma development in vivo	In progress	M 26.11	39

Additional workpackages (WPs 27-32)

WP 27

Systematic analysis of gene expression in primary human tumors. (Agendia)

Agendia is a SME which identifies gene expression signatures that are of diagnostic and prognostic value for the treatment of cancer. Agendia will perform gene expression analysis using DNA microarrays on several human tumour series including melanoma, lymphoma and colon cancer. Prognostic gene expression signatures will be obtained that predict disease outcome in these forms of cancer. Genes that are part of such prognostic signatures are candidate targets for drug development. A database will be generated that harbours Agendia's gene expression data, its prognostic signatures and the gene expression data from microarray studies from the public domain. This will allow participants from the consortium to quickly search in which form of human cancer the genes they identify in their genetic screens are expressed.

In 2006, we have worked on a prognostic gene expression signature for early stage Non small cell lung cancer (NSCLC). We started off by establishing an academic consortium of 5 cancer centers across Europe: two in the Netherlands, one in Germany and two in Poland. We identified in these centers 322 NSCLCs from patients that met the following criteria: no prior malignancy, no metastasis, stage 1 or 2, >2 years follow up, frozen tumor sample available. From this set we selected 108 samples for initial profiling on 44K complex microarrays. Supervised classification identified a 54 gene signature that identifies a high risk subgroup in the patient population. The Kaplan-Meier survival curves for both the high risk and the low risk patients are given in Figure 27-1, left panel. When combined with the stage classification of the tumor, the 54-gene signature has significant ability in both stage 1 and stage 2 NSCLC to predict prognosis (Figure 27-1, right panel). Our work on gene expression profiling of older age breast cancer patients is completed and a classifier of 100 genes has been built (Figure 2). We also finalized the gene profiling studies on melanoma and had this completed on schedule in Q3 of 2005.

We have acquired state of the art software (Rosetta's "Resolver") to build the database of gene expression signatures from the public domain. We have spent more time than anticipated to implement the Resolver software in 2006. It should be fully operational first quarter of 2007, after which we will upload our sets of gene expression signatures.

Third Annual Periodic Activity Report (2006)

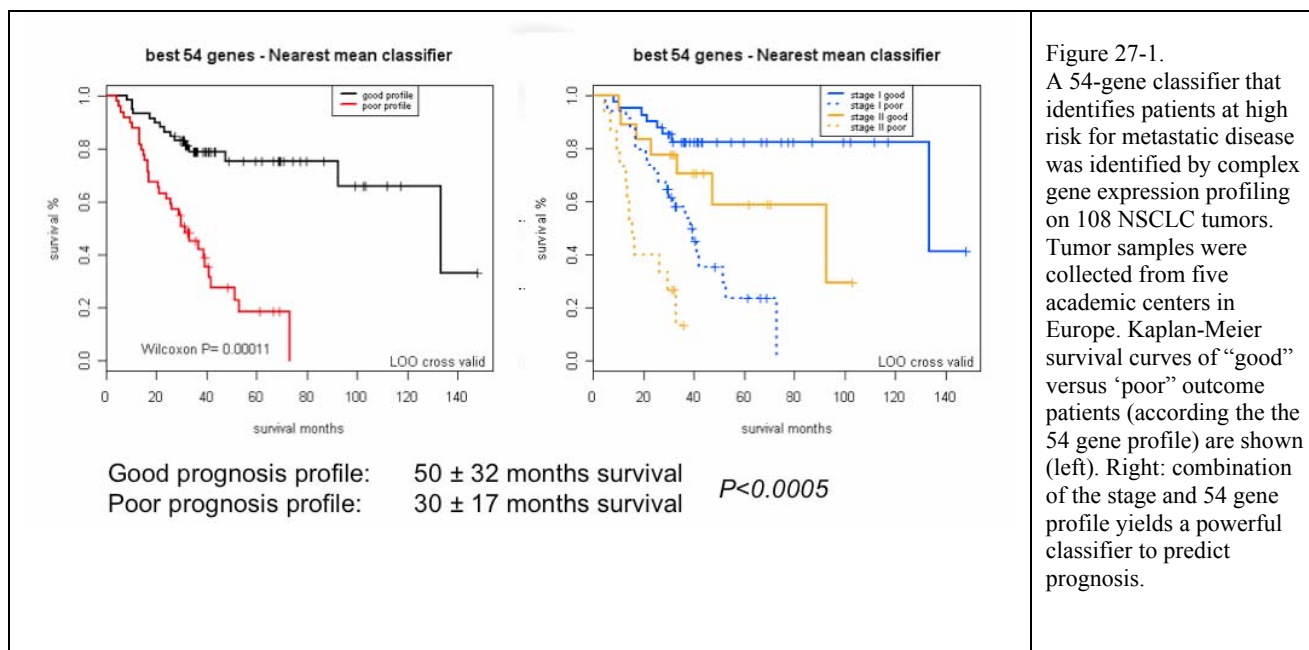


Figure 27-1. A 54-gene classifier that identifies patients at high risk for metastatic disease was identified by complex gene expression profiling on 108 NSCLC tumors. Tumor samples were collected from five academic centers in Europe. Kaplan-Meier survival curves of “good” versus “poor” outcome patients (according to the 54 gene profile) are shown (left). Right: combination of the stage and 54 gene profile yields a powerful classifier to predict prognosis.

Accumulative list of tasks, deliverables and milestones in WP27 (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 27.1	Identify prognostic gene expression signatures for three types of human cancer	Achieved	M 27.1	18
	T 27.2	Building of database of gene expression signatures from public domain microarray data sets.	Achieved	M 27.2	18
Update 12-30	T 27.3	Identify prognostic gene expression signature for lung cancer.	In progress	M27.3	36
	T 27.4	Extend the existing database of gene expression signatures from public domain microarray data sets.	In progress	M24.4	48
	T 27.5	Customize Resolver software and hardware for storing database of microarray data	In progress	M27.5	36
Update 25-42	T 27.6	Validate prognostic signature for lung cancer	In progress	M27.8	48

WP 28

Morphochem – workpackage ended

WP29
Management activities
(UKBH)

See management report.

WP 30
CGTM activities
(GEM)

Objectives

1. Complement the RTD aspect, and its networking of major laboratories in the cancer field, by the provision of training to young scientists throughout the EC.
2. Coordination group for Training and Mobility (CGTM) will oversee all training aspects of the consortium including
 - a) the recruitment process (if necessary at this stage), to ensure scientific excellence in the WPs carried out under the RTD sections of the proposal, by the recruitment of the best candidates in an inclusive and open manner (Task 1).
 - b) organising symposia.
 - c) proposing training workshops to the executive committee.
 - d) prepare an application as a Marie-Curie-Training Network for the Consortium and/or help with individual applications.
 - e) sensitise the consortium to any gender matters arising in recruitment, staff management, and, potentially, research topics. If necessary, CGTM will recruit external experts to the symposium.

Cumulative list of tasks, deliverables and milestones in the WP (months 1 - 42):

	No.	Task name	Status	Associated milestone	Milestone date
Original	T 30.1	Recruitment of qualified personnel to the EC funded positions. CGTM will organize coordinate advertisement in international journals and oversee recruitments (if necessary at this stage)	achieved/in progress	M 30.1	12-48
	T 30.2	Symposia: CGTM will help organising two symposia in the four years. The first symposium has been arranged in January 2006, the second in February 2007	achieved	M 30.2	24 and 36
	T 30.3	Training workshops/Intensive working groups: CGTM will propose a series of teaching workshops/small meetings on defined topics of the methodologies in WP 1-6 to the executive committee. Individual participants (institutions attendees) are responsible for organization. Two workshops have been held at NKI, one has been held in Marburg; intensive working groups (IMT-NKI2) and (Pasteur-NKI2)	achieved/in progress	M 30.3	12, 24, and 36; 15 and 17
	T 30.4	prepare an application for a Marie-Curie-Training Network for the Consortium and/or help with individual applications (first stage) (submitted on Sept 27 th , 2005; approved by the EC in the Dec round of selection)	achieved	M 30.4	20
	T 30.5	prepare the second stage application for a Marie-Curie-Training Network for the Consortium (due Feb 14 th , 2006). The application has been filed but turned down (B)	achieved	M 30.5	25
	T 30.6	Monitor Gender Issues: CGTM will monitor gender issues at multiple levels.	in progress	M 30.6	48

WP 31

Management activity, Web presence (UNIMAR)

www.imt.uni-marburg.de/intact/

The website was updated once in 2006. It has been used successfully to announce and organize through links the HCS Course that was run in Marburg in November 2006course and the INTACT symposium during the year. It will be updated again during 2007 to include the annual report (password protected).

Objectives (due to revised strategy)

No change of Strategy has occurred

Accumulative list of tasks, deliverables and milestones in the WP 31 (months 1 - 30):

	No.	Task name	Status	Associated milestone	Milestone date
50	T31.1	Build-up and maintain INTACT website.	Achieved	M 31.1	6-48

WP 32

Study of synthetic lethality on Myc overexpressing cell lines (Vichem)

The concept of synthetic lethality predicts that a class of genes exist that is required for the survival of cells that carry oncogenic mutations but that is dispensable for the growth of normal cells. Identification of synthetic lethal interactions will be of key importance for the identification of new and more powerful classes of drug targets.

Activation of the c-Myc oncogene induces apoptosis in virtually all cell types that have been tested. For tumours with high Myc levels to emerge, secondary mutations must therefore have occurred that disable pro-apoptotic programs that would otherwise be activated by c-Myc. Another example, inhibition of a gene that shows a synthetic lethal interaction with loss of p53 will only be toxic in cells harbouring a mutation in p53, making it a cell-specific drug target. Consequently, inhibition of such genes in normal cells harbouring wild type p53 is much less toxic, providing a therapeutic window for cancer treatment.

Identification of synthetic lethal drug targets and compounds specifically inducing cell death in cancer cells carrying certain mutations (in particular c-myc overexpression and p53 mutation) is a major focus of the INTACT project.

Other consortium members utilize RNAi libraries to identify synthetic lethal molecular targets (e.g. WP12) in p53 mutant or c-Myc overexpressing cell lines, VICHEM is using its proprietary Nested Chemical Library (NCL) technology to chemically screen isogenic cellular models in proliferation and cell death assays. NCL was built around 108 chemical core structures containing a highly diverse collection of small molecular weight kinase inhibitory compounds. Subsequently, the cell lines are screened using the Chemical Validation Library (CVL) and the Extended Validation Library (EVL) which also include our novel proprietary compounds. At the end of the screening process hit compounds will be presented for further characterization and pharmacophore model based QSAR analysis, and drug target molecules binding to these compounds will be identified by Vichem's proprietary KINATOR technology.

Cellular Bioassays for drug screening

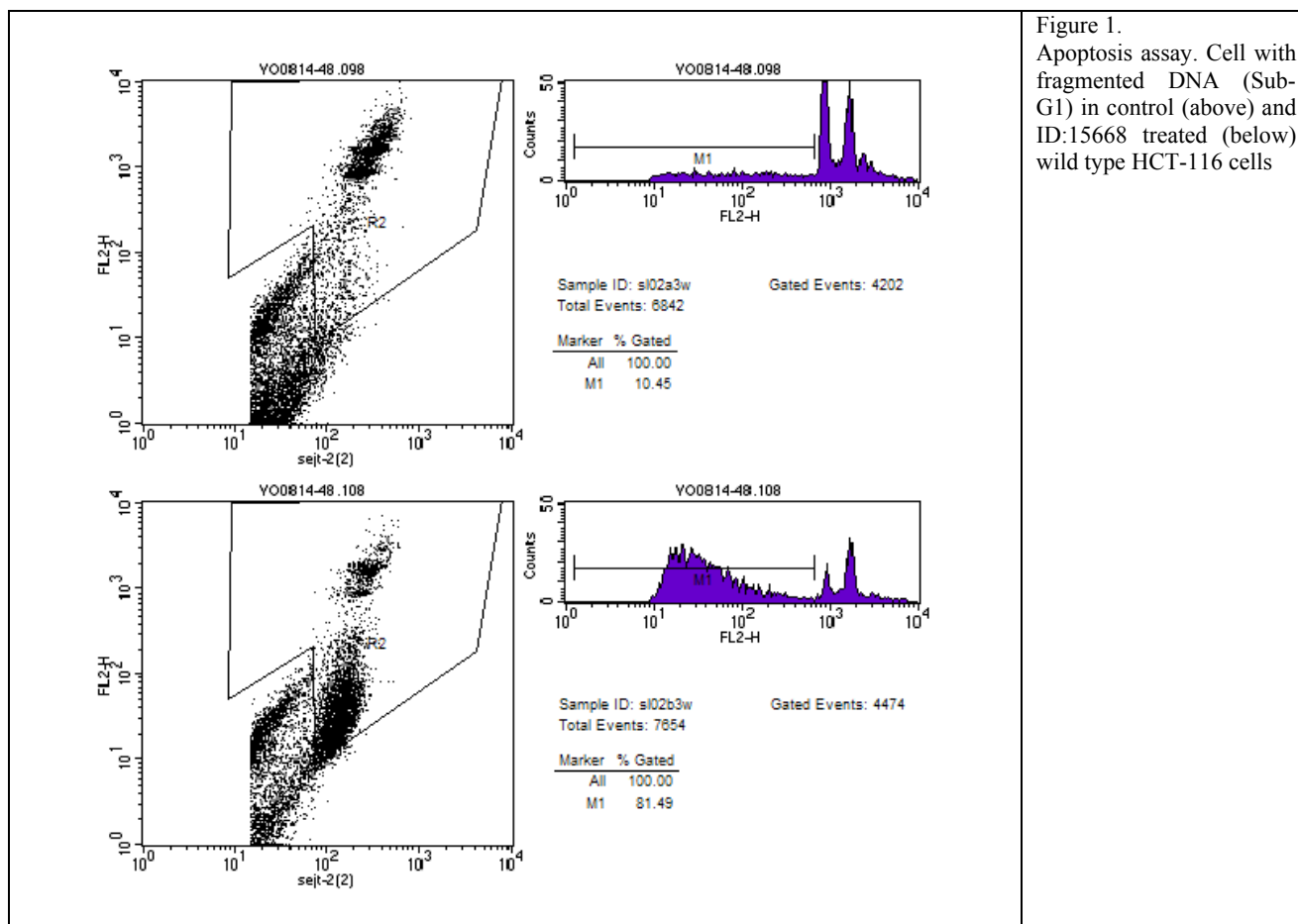
Although, originally the plan was to chemically screen c-Myc overexpressing cells for synthetic lethal drug targets in this WP, however we have decided to start our work first on the p53 synthetic lethal model. Our reasoning was that p53 is the most frequently affected gene in human cancer and loss of p53 function is more cancer specific than c-Myc overexpression which may overlap with highly proliferating normal cells. Also, there is a much greater competition in the research field on the biology of c-Myc related synthetic lethality. Therefore, we may maximize the probability to identify a novel proprietary cancer specific drug target and preclinical lead compound before the end of this project.

We have optimized the cell culture conditions of the p53 null and wild type isogenic HCT116 colon carcinoma cell lines. We have frozen down large number of vials of low passage cells to use uniform passage number cells throughout the project. We have tested the doubling times, the sub-G1 apoptosis assay to optimally design the cellular assay.

Cell culture and experiments were carried out in RPMI-1640 (Biowhittaker) medium supplemented with 10% FCS (Biowhittaker) and L-Glutamine (Sigma) at 37°C, in an atmosphere of 95% air and 5% CO₂. For all experiments cells growing in the exponential phase were used. Plastic materials were purchased from Sarstedt. Cells were plated at a density of 105cells/ml in 24-well plates. After overnight attachment cells were incubated in the presence or absence of various treatments. Stock solutions were made from all compounds in DMSO (dimethylsulfoxide, from Sigma) at concentrations that the final dilutions contained less than 0,2% DMSO.

At the end of incubation times, cells were harvested (both floating and attached) and fixed in 70% ethanol (-20°C) for 30 min, and stored at -20°C until analysis. Fragmented DNA was extracted by using alkaline

buffer (200 mM di-sodiumphosphate, pH 7.8, adjusted with 200 mM Na-citrate) supplemented with 100 ug/ml DNase-free RNaseA (Sigma). Samples were left at RT for 20 min followed by the addition of 5 ul propidium iodide (final concentration 10 ug/ml). After a further 10 min, samples were measured with a flow cytometer (FACScan, Becton-Dickinson) and cell cycle and proportion of sub-G1 (apoptotic) cells were calculated. (Figure 1)



First screening results

We have already screened the first 20 compounds of the NCL which have been synthesized with chemical linkers ready for subsequent KINATOR technology. Further 40 compounds of NCL without chemical linkers were screened as well.

Both P53 null and wild type cells were incubated in parallel in the absence or presence of the test compounds at the concentration of 10^{-6} M for 72 hours in duplicates and subsequently processed for apoptosis assay as described above. The increase in the ratio of subG1 population in the treated samples in comparison to the control values were calculated and expressed as specific apoptotic index. (Figure 2-4.)

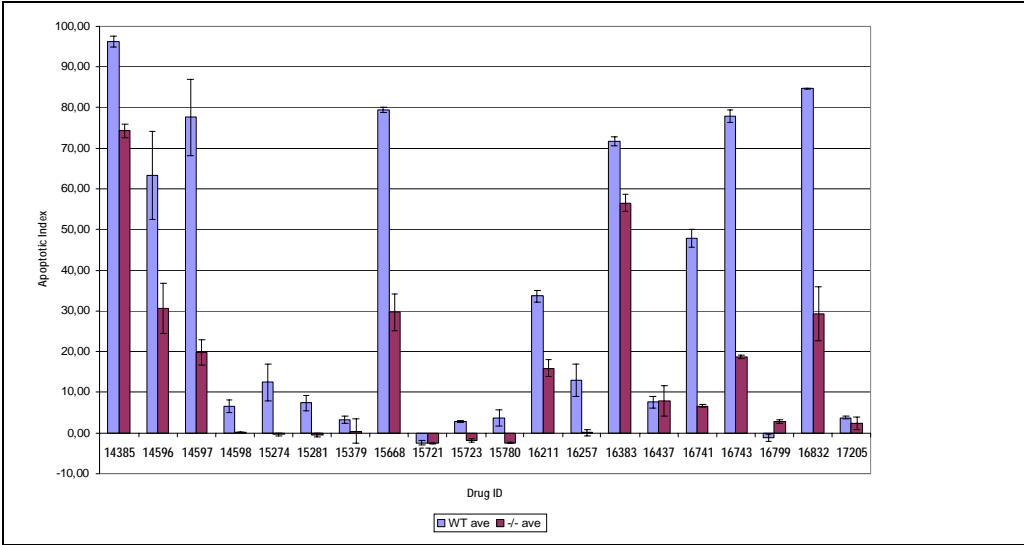


Figure 2.
Screening results of
KINATOR
labelled
compounds

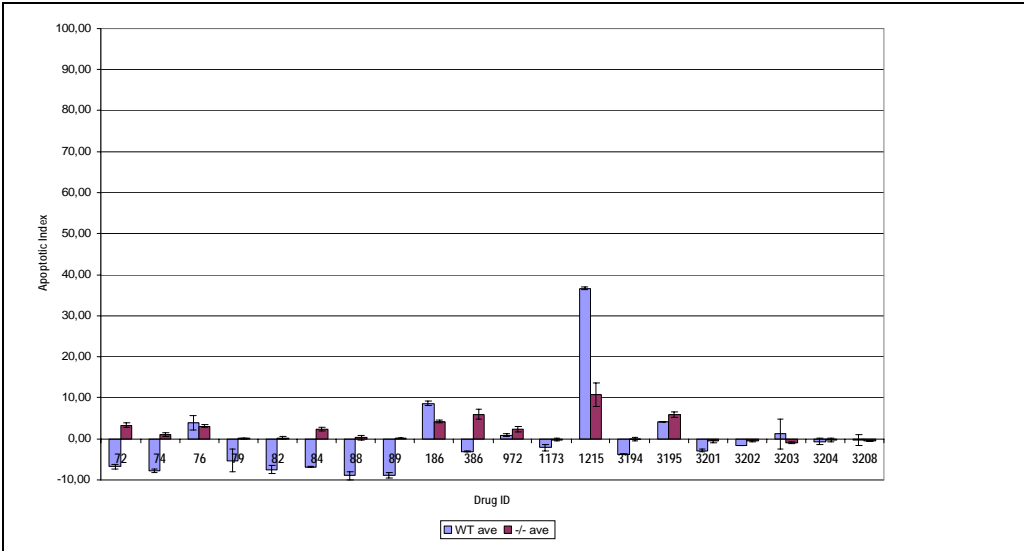


Figure 3.
Screening results of non-
labelled compounds

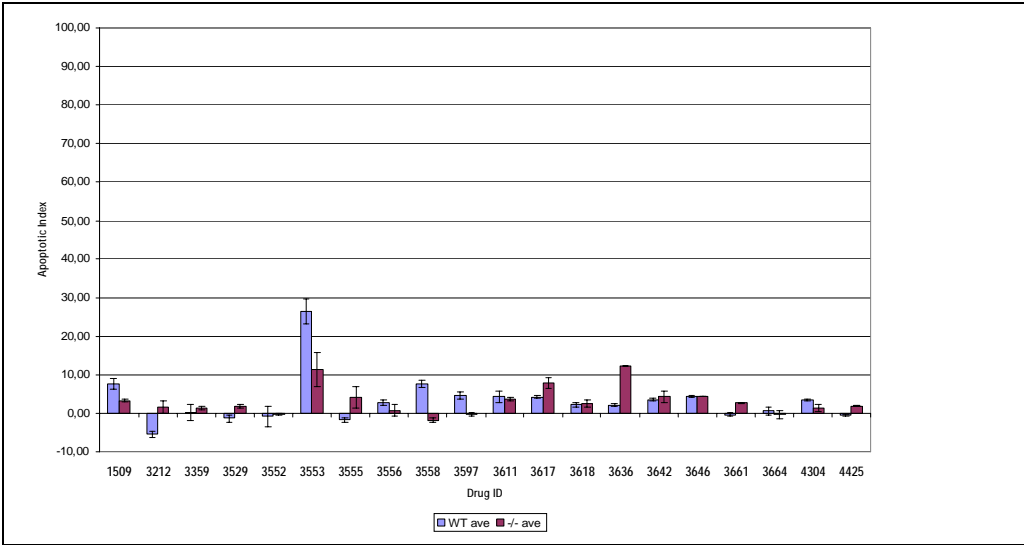


Figure 4.
Screening results of non-
labelled compounds

As expected, p53 null cell were generally more resistant to apoptosis inducing effect of kinase inhibitors. 7 out of 20 kinator labelled compounds induced more than 50% apoptosis at this concentration in the wild type p53 cells. However, we identified 2 compounds (ID14385 and ID16383) which induced more than 50% apoptosis in p53 null cells. Therefore these compounds were screened repeatedly, in four parallel measurements.

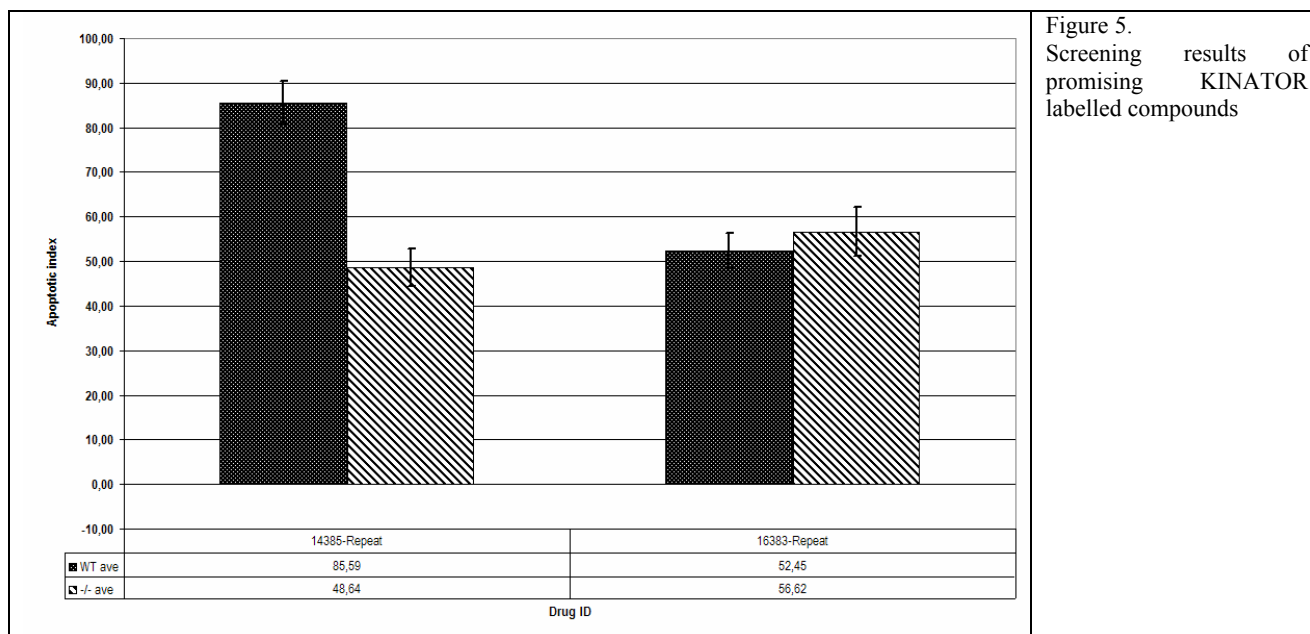


Figure 5. Screening results of promising KINATOR labelled compounds

Drug compounds ID14385 and ID16383 induced more than 50% apoptosis in both cell lines again. (Figure 5) ID16383 showed higher specific apoptotic index in p53 deficient mutant cell line, although difference between mutant and wild type is very slight. Therefore further screening of compound ID16383 is needed, efficiency must be optimized in time and in concentration as well.

However, to find new potential targets further screening of the non-labeled compounds of chemical library is progress.

Cytotoxicity and Viability Assay

We used Promega MultiTox-Fluor Multiplex Cytotoxicity Assay as an alternative method to measure cytotoxicity and viability of wt and -/- cells. The Promega MultiTox-Fluor Multiplex Cytotoxicity Assay is a single-reagent-addition, homogeneous, fluorescent assay that measures the number of live and dead cells simultaneously in culture wells.

The assay simultaneously measures cell viability and cytotoxicity by detecting two distinct protease activities. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant peptide substrate (GF-AFC Substrate). The substrate enters intact cells where it is cleaved to generate a fluorescent signal proportional to the number of living cells. This live-cell protease activity marker becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium. A second, non-cell-permeant, fluorogenic peptide substrate (bis-AAF-R110 Substrate) is used to measure dead-cell protease activity that has been released from cells that have lost membrane integrity.

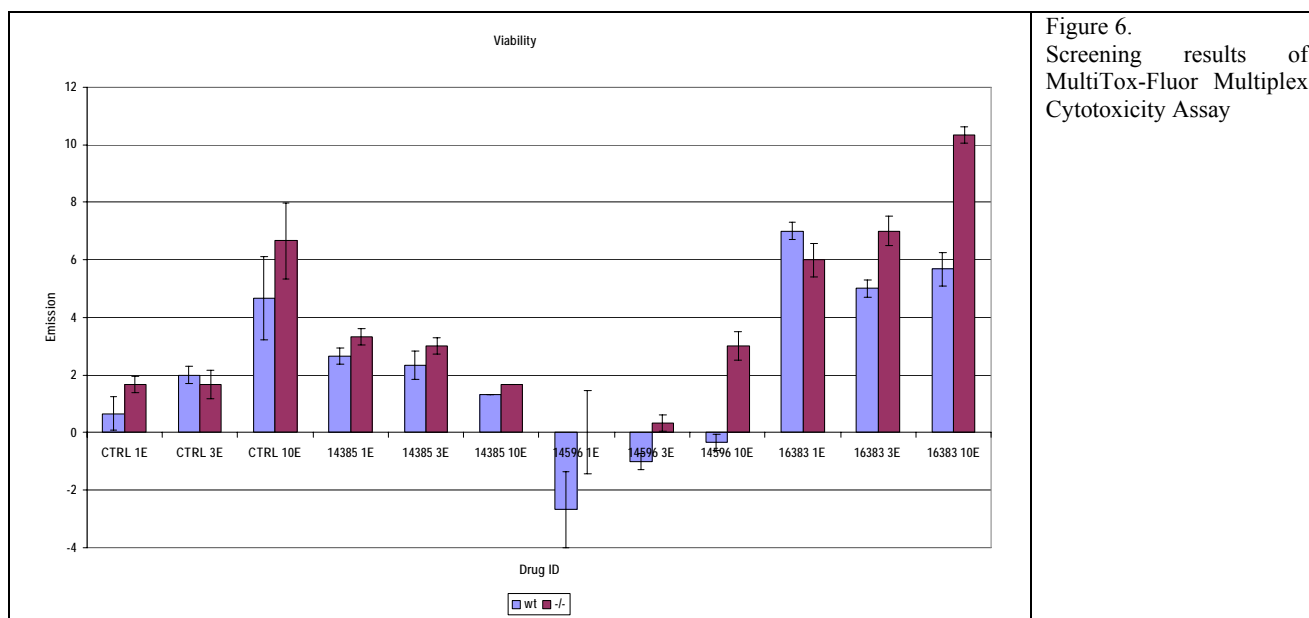
Known number of cells were suspended in 100 μ l culture medium (RPMI-1640 incl. 10% FBS) and placed on 96 well plate. (For plate configuration see Table 1.) After 16 hours of adhesion cells were treated with target molecules diluted in DMSO ($C = 0.005 \text{ mol} \cdot 10^{-1} \text{ l}$ $V = 0.2 \text{ ul}$). 100 μ l of MultiTox-Fluor Multiplex Cytotoxicity Assay were pipetted to each well 24 hours after treatment. 96 well plate was incubated 40 min at 37°C. Viability and cytotoxicity was measured with BioTek Synergy multiplate reader (Table 2).

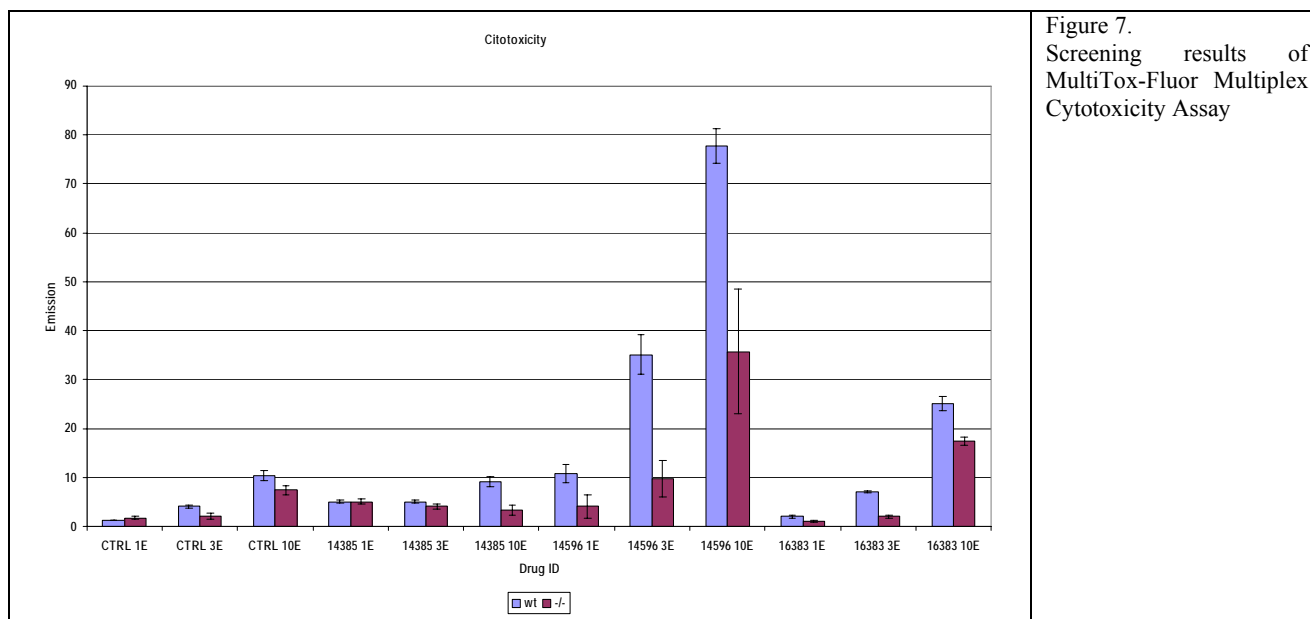
Third Annual Periodic Activity Report (2006)

BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Table 1. Assembly of plate. CL1: wt CL2: -/- CTRL: control DRUG1: 14305, DRUG2: 14596 DRUG3: 16383 Number of cells: 1E=1000 3E=3000 10E=10000
CL1 1E CTRL	CL1 3E CTRL	CL1 10E CTRL	CL1 1E DRUG1	CL1 3E DRUG1	CL1 10E DRUG1	CL1 1E DRUG2	CL1 3E DRUG2	CL1 10E DRUG2	CL1 1E DRUG3	CL1 3E DRUG3	CL1 10E DRUG3	
CL1 1E CTRL	CL1 3E CTRL	CL1 10E CTRL	CL1 1E DRUG1	CL1 3E DRUG1	CL1 10E DRUG1	CL1 1E DRUG2	CL1 3E DRUG2	CL1 10E DRUG2	CL1 1E DRUG3	CL1 3E DRUG3	CL1 10E DRUG3	
CL1 1E CTRL	CL1 3E CTRL	CL1 10E CTRL	CL1 1E DRUG1	CL1 3E DRUG1	CL1 10E DRUG1	CL1 1E DRUG2	CL1 3E DRUG2	CL1 10E DRUG2	CL1 1E DRUG3	CL1 3E DRUG3	CL1 10E DRUG3	
CL2 1E CTRL	CL2 3E CTRL	CL2 10E CTRL	CL2 1E DRUG1	CL2 3E DRUG1	CL2 10E DRUG1	CL2 1E DRUG2	CL2 3E DRUG2	CL2 10E DRUG2	CL2 1E DRUG3	CL2 3E DRUG3	CL2 10E DRUG3	
CL2 1E CTRL	CL2 3E CTRL	CL2 10E CTRL	CL2 1E DRUG1	CL2 3E DRUG1	CL2 10E DRUG1	CL2 1E DRUG2	CL2 3E DRUG2	CL2 10E DRUG2	CL2 1E DRUG3	CL2 3E DRUG3	CL2 10E DRUG3	
CL2 1E CTRL	CL2 3E CTRL	CL2 10E CTRL	CL2 1E DRUG1	CL2 3E DRUG1	CL2 10E DRUG1	CL2 1E DRUG2	CL2 3E DRUG2	CL2 10E DRUG2	CL2 1E DRUG3	CL2 3E DRUG3	CL2 10E DRUG3	
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	

	Excitation (nm)	Emission (nm)	Table 2. Assay and reader configuration
Viability optimum (Promega)	400	505	
Measured viability (filter in BioTek)	360 / 40	528 / 20	
Citotoxicity optimum (Promega)	485	520	
Measured citotoxicity (filter in BioTek)	485 / 20	528 / 20	

Measured results (Figure 6-7) are not significant since measurement was made one time only for testing the method. Results show dependence on cell number and drug treatment. Results show also tendency in viability and citotoxicity. Results prove that Promega MultiTox-Fluor Multiplex Cytotoxicity Assay could be used as an alternative, cost-effective method after regularly optimisation of cell number, treatment and incubation time.





Comparison of the whole genome kinase expression profile in p53 +/+ and p53-/- HCT116 colon carcinoma cell lines

As a parallel alternative approach to the chemical screening, we have searched for synthetic lethal drug targets for our kinase inhibitor chemical library by the expression profiling of the whole human “kinome” of 518 kinases in both isogenic cell lines.

We have used the novel technology of “Nanoliter Multiplex Real-Time PCR system” (BioTrov-Avidin) which allows mid-throughput expression studies in small amount of nucleotid samples in higher exactitude than any other microarray systems. Total RNA was extracted from cells growing the logarithmic phase by QIAGEN RNeasy Kit and subsequently the quantitative real-time PCR was carried out in nanoliter volumes on a highly integrated silicon microchamber array of human kinases in quadriplex format.

We have identified 24 kinases which are expressed more than 3 times higher level in the p53 null cells than in the parent p53 wild type cells (Table 3.).

Assay. Ref Seq ID	Assay. Gene Name	Assay.Fxn1 Cat	MUT Ave	MUT stdev	WT Ave	WT stdev	WT-MUT	MUT/ WT
NM_002314	LIMK1	protein Ser/Thr kinase activity	19.71	0.32	21.25	0.89	1.54	2.91
NM_033015	FASTK	protein Ser/Thr kinase activity	19.48	0.00	21.06	####	1.58	2.99
NM_001345	DGKA	diacylglycerol kinase activity	20.10	0.00	21.69	0.84	1.60	3.02
NM_002498	NEK3	protein Ser/Thr kinase activity	20.02	0.54	21.65	0.35	1.63	3.10
NM_001347	DGKQ	diacylglycerol kinase activity	21.47	1.02	23.12	0.68	1.65	3.13
NM_004935	CDK5	cyclin-dependent protein kinase activity	18.38	0.00	20.04	0.58	1.65	3.15
NM_005160	ADRBK2	G-protein coupled receptor kinase activity	18.41	0.14	20.07	0.40	1.66	3.16
NM_000167	GK	glycerol kinase activity	21.16	0.15	22.84	0.76	1.68	3.21
NM_005232	EPHA1	TM-receptor prot. Tyr-kinase activity	19.91	2.20	21.62	0.45	1.70	3.25
NM_002611	PDK2	protein kinase activity	17.93	0.48	19.65	0.21	1.72	3.30
NM_022963	FGFR4	fibroblast growth factor receptor activity	20.35	1.69	22.08	1.17	1.74	3.33
NM_006343	MERTK	TM-receptor prot. Tyr-kinase activity	20.68	0.70	22.49	1.91	1.81	3.51
NM_007181	MAP4K1	protein serine/threonine kinase activity	24.11	0.00	25.93	1.35	1.82	3.53
NM_005881	BCKDK	protein kinase activity	17.73	0.08	19.57	0.10	1.84	3.59
NM_004333	BRAF	protein kinase activity	18.03	0.05	19.91	1.75	1.89	3.70

Table 3.
List of kinase genes in -/- mutant p53 cell line showing higher expression level

Third Annual Periodic Activity Report (2006)

NM_145259	ACVR1C	type I transforming growth factor beta receptor activity	19.55	0.58	21.58	0.30	2.03	4.09
NM_002739	PRKCG	calcium-dependent protein kinase C activity	23.12	####	25.19	0.47	2.07	4.20
NM_005391	PDK3	protein kinase activity	19.12	0.55	21.28	1.68	2.16	4.48
NM_016203	PRKAG2	SNF1A/AMP-activated protein kinase activity	21.20	0.75	23.41	1.16	2.21	4.62
NM_003551	NME5	nucleoside-diphosphate kinase activity	22.39	0.68	24.69	1.18	2.29	4.91
NM_000215	JAK3	protein-tyrosine kinase activity	23.43	0.69	26.04	0.35	2.61	6.11
NM_020629	RET	cAMP-dependent protein kinase activity	22.86	0.00	26.77	1.43	3.91	15.04
NM_020630	RET	cAMP-dependent protein kinase activity	24.41	1.32	28.59	1.45	4.17	18.04
NM_005198	CPT1B	choline kinase activity	15.73	0.00	20.41	0.71	4.69	25.77
NM_006252	PRKAA2	cAMP-dependent protein kinase activity	19.61	0.28	28.31	####	8.71	418

The most promising targets are the proto-oncogene receptor tyrosine kinase RET, which is expressed more the 16-times in the p53 null cells. The other known cancer related signal transduction therapy target molecule is the JAK3.

Interestingly, many of the genes showing higher expression level in -/- mutant p53 cell line is involved in the long-chain fatty acid beta-oxidation pathway including the carnitine palmitoyltransferase-1 (CPT-1) which regulates the level of ceramide, an important mediator of apoptotic pathways (Figure 8).

<p style="text-align: center;">NM_020629 NM_020630 RET; ret proto-oncogene</p> <p>This gene, a member of the cadherin superfamily, encodes one of the receptor tyrosine kinases, which are cell-surface molecules that transduce signals for cell growth and differentiation. This gene plays a crucial role in neural crest development, and it can undergo oncogenic activation in vivo and in vitro by cytogenetic rearrangement. Mutations in this gene are associated with the disorders multiple endocrine neoplasia, type IIA, multiple endocrine neoplasia, type IIB, Hirschsprung disease, and medullary thyroid carcinoma. Two transcript variants encoding different isoforms have been found for this gene.</p> <p style="text-align: center;">NM_000215 JAK3, Janus kinase 3</p> <p>The Janus family of tyrosine kinases (JAKs) has emerged as a promising target for therapeutic agents. JAKs are involved in pathways which help regulate cellular functions in the lympho-hematopoietic system critical for cell proliferation and cell survival. JAKs are abundantly expressed in primary leukemic cells from children with acute lymphoblastic leukemia (ALL) and are involved in signals regulating apoptosis. Two recently reported dimethoxyquinazoline compounds, WHI-P131 and WHI-P154 (Hughes Institute), were found to inhibit JAK3 but not JAK1 or JAK2. The high potency and selectivity of WHI-P131 for JAK3 makes it a promising candidate for new treatment strategies against ALL, the most common form of childhood cancer. In addition to its antileukemic properties, WHI-P131 also shows clinical potential for the treatment of mast cell-mediated immediate hypersensitivity reactions and allergic disorders, including asthma, as well as immunosuppression of alloimmune and autoimmune disorders.</p> <p style="text-align: center;">NM_005198 CPT1B; carnitine palmitoyltransferase 1B, choline kinase activity</p> <p>This enzyme is the rate-controlling enzyme of the long-chain fatty acid beta-</p>	<p>Figure 8. Screening results of MultiTox-Fluor Multiplex Cytotoxicity Assay</p>
---	---

Third Annual Periodic Activity Report (2006)

oxidation pathway mitochondria and is required for the net transport of long-chain fatty acyl-CoAs from the cytoplasm into the mitochondria in muscle. There are results showing that a carnitine palmitoyltransferase-1 (CPT-1) inhibitor up-regulated the ceramide and pro-apoptotic BNIP3 levels in these cells. Inhibition of FAS in breast cancer cells causes accumulation of malonyl-CoA, which leads to inhibition of CPT-1 and up-regulation of ceramide and induction of the proapoptotic genes BNIP3, TRAIL, and DAPK2, resulting in apoptosis. Treatment of tumor cells with FAS siRNA in the presence of a ceramide synthase inhibitor abrogated the up-regulation of BNIP3 and inhibited apoptosis. (Cancer Res. 2006 Jun 1;66(11):5934-40.)	
--	--

Objectives (revised due to change of strategy)

- Identify novel small molecular weight synthetic lethality inducing compounds of wild type and p53 null isogenic HCT-116 colon carcinoma cell lines
- Hit finding and lead selection.

	No.	Task name	Status	Associated milestone	Milestone date
Update 25-42	T 32.1	Installation of cellular inhibitory assay	Achieved	M 32.1	30
	T 32.2	Testing of NCL compounds.	In progress	M 32.2	48
	T 32.3	Quantitative structure-activity relationships generation based on inhibition date.	Not started yet	M 32.2	48

Section 3 – Consortium management

The tables in this section describe the status and progress of how specific deliverables and milestones are met, and a front lined barchart visualizes how the tasks are progressing in relation to the planning. Tasks, deliverables, and milestones defined in Annex 1, first annual activity report are marked with black, items defined in the period months 12-30 are marked with blue, and items defined in the period months 25-42 are marked with green.

List of deliverables (months 1-42)

	No. (prev. no.)	Deliverable name	WP	Date due	Actual/ Forecast, delivery date	Estimated indicative person- months*)	Used indicative person- months*)	Lead contractor
Original	D 4.1 (31)	Concentrated high titer viral supernatants	4	9	9	9	12	IEO
	D 4.2 (73)	Microarrays of retrovirus on glass slides	4	18	24	18	24	IEO
Update 13-30	D 4.3	Array of normalized viral clones of the kinase library on chip	4	30	48/36	9	6	IEO
	D 4.4	Method for efficient RNA interference in human primary and cancer cells in array format	4	24-30	36	9	6	IEO
Update 25-42	D 4.5	Optimized nanostructured TiO ₂ substrate for viral immobilization	4	48	36	9	9	IEO
	D 4.6	Methods for immunofluorescence assays on glass slides	4	48	36	9	9	IEO
Original	D 5.1	A set of tested vectors available for distribution to consortium members.	05	24	36	8	8	NKI1
	D 5.2	Set of detailed protocols for infection of pre-implantation embryos.	05	21	24	8	8	NKI1
	D 5.3	Transgenic line with generally expressed tet-repressible transactivator.	05	24	24	4	4	NKI1
Update 12-30	D 5.4	Validated transgenic reporter mouse line for measuring p53	05	30	42	8	8	NKI1
Original	D6.1	Validated siRNAs against components of the Wnt pathway. LS174T cell lines with inducible siRNA cassettes	6	18	30	8	8	ISREC2
	D6.2	A single vector lentiviral system for inducible expression of siRNAs	6	18	30	5	5	ISREC2

Third Annual Periodic Activity Report (2006)

Update 12-30	D 6.1	Validated siRNAs against components of the Wnt pathway. LS174T cell lines with inducible siRNA cassettes	6	18	30	8	8	ISREC2
	D 6.2	A single vector lentiviral system for inducible expression of siRNAs	6	18	30	5	5	ISREC2
	D 6.3	Data on induced knock-down of critical components in the canonical β -catenin signaling pathway in grafted LS174T cells	6	24	24	6	6	ISREC2
	D 6.5	Protocols for infection and orthotopic grafting of primary tumours established	6	27	30	6	6	ISREC2
	D 6.6	Role of signaling components assessed in orthotopically grafted primary tumour cells	6	30	33	9	9	ISREC2
Update 25-42	D 6.1	Validated siRNAs against components of the Wnt pathway. LS174T cell lines with inducible siRNA cassettes	6	18	30	8	8	ISREC2
	D 6.2	A single vector lentiviral system for inducible expression of siRNAs	6	18	30	5	5	ISREC2
	D 6.3	Data on induced knock-down of critical components in the canonical β -catenin signaling pathway in grafted LS174T cells	6	24	24	6	6	ISREC2
	D 6.4	Protocols for infection and orthotopic grafting of primary tumours established	6	27	30	6	6	ISREC2
	D 6.5	Role of signaling components assessed in orthotopically grafted primary tumour cells	6	30	33	9	9	ISREC2
Original	D 7.1 (42)	Cell lines with CDC25A-GFP fusions	7	6	6	6	3	IEO1
	D 7.2 (43)	Novel ATM-ATR/CHK1-CHK2-specific genes, which provide new leads for cancer intervention strategies	7	12	12	6	1	IEO1
	D 7.3 (80)	Initial biological characterization of genes identified in the RNAi screens	7	18	18	6	3	UKBH
	D 7.4 (81)	Data regarding the expression of mediators of the G2/M checkpoint in cancer	7	18	30	6	6	UKBH
Update 12-30	D 7.5	A suitable cellular system to screen for inhibitors of the G2/M DNA damage checkpoint	7	12	12	6	6	UKBH
	D 7.6	Candidate genes required for the G2/M checkpoint	7	18	18	6	6	UKBH
	D 7.7	Mediators of the G2/M checkpoint	7	24	24 (in part)	6	6	UKBH

Third Annual Periodic Activity Report (2006)

Update 25-42	D 7.8	Establishment and performance of HCS	7	36	42	6	6	UKBH
	D 7.9	Identification of proteins bound to mediators of G2/M checkpoint	7	36	36	6	6	UKBH
Original	D 8.1 (44)	Double knockout mice for telomerase and telomere-binding proteins	8	12	6	7,5	7,5	CNIO1
	D 8.2 (11)	New RNAi retroviral vectors to telomerase and different telomere-binding proteins	8	6	6	7,5	7,5	CNIO1
	D 8.3 (82)	Identification of new activities important for telomere capping and telomere signalling	8	18	18	6	6	CNIO1
	D 8.4 (83)	New mouse models and cellular systems	8	18	18	7,5	7,5	CNIO1
Update 12-30	D 8.5	Cell line suitable for genetic screens aimed at the identification of genes that bypass cell death caused by telomere dysfunction	8	15	15	2	2	CNIO1
	D 8.6	Candidate genes that bypass cell death induced by telomere dysfunction	8	24	30	3	0	CNIO1
	D 8.7	Mediators of telomere-driven apoptosis	8	27	33	5	0	CNIO1
	D 8.8	Regulators of telomere length in different models of telomere damage	8	30	36	5	0	CNIO1
Original	D 9.1 (84)	Identification of new targets for tumour progression and metastatic disease	9	18	24	10	10	CNIO1
	D 9.2 (12)	New RNAi retroviral vectors	9	6	24	10	10	CNIO1
	D 9.3 (85)	New mouse models	9	18	18	10	10	CNIO1
Update 12-30	D 9.4	High-throughput Q-FISH telomere length determination method	9	15	18	3	3	CNIO1
	D 9.5	Telomerase reporter cell line	9	15	15	1	1	CNIO1

Third Annual Periodic Activity Report (2006)

	D 9.6	Genes that regulate telomere length and/or telomerase activity in a human tumour cell line	9	24	42	5	1	CNIO1
	D 9.7	Identification of novel regulators of telomere length and/or telomerase activity with relevance to telomere-driven tumourigenesis and metastasis in vivo	9	30	48	6	0	CNIO1
Original	D 10.1	Human epithelial cell lines with tet-repressor	10	6	6	6	6	BICR
	D 10.2 (1)	GFP/RFP reporter constructs for p53 target genes p21 and Bax	10	6	6	6	6	BICR
	D 10.3 (2)	Reporter cell lines that stably express p53 reporter constructs	10	12	12	6	6	BICR
	D 10.4 (2)	Reporter cell lines that stably express p53 reporter constructs and tet-repressor	10	12	12	6	6	BICR
	D 10.5 (3)	Cells lines which are suitable for the performance of the screens	10	15	15	3	3	BICR
	D 10.6 (4)	Novel p53 pathway-specific genes from RNAi screen	10	21	39	6	6	BICR
	D 10.7 (5)	Data on initial characterisation of respective gene products	10	24	48	6	6	BICR
	D 10.8 (6)	Data on validation of identified genes in tissue culture	10	24	48	6	6	BICR
Update 12-30	D 10.9	Luciferase-based cellular systems to screen for p53 pathway regulators	10	30	33	18	21	BICR
Update 25-42	D 10.6	Novel p53 pathway-specific genes from RNAi screen	10	21	39	6	9	BICR
	D 10.7	Data on initial characterisation of respective gene products	10	24	48	6	9	BICR
Original	D 11.1	PUMA-GFP reporters	11	12	12		3	BICR
	D11.2	Generation of reporter cell lines	11	15	15		3	BICR

Third Annual Periodic Activity Report (2006)

	D 11.3	Characterization of cell lines	11	18	25	10		BICR
	D11.4	Novel PUMA pathway genes	11	21	30	5		BICR
	D11.5	Initial biological characterization of genes identified in the RNAi screens	11	27	36	6		BICR
	D11.6	Validation of identified genes in tissue culture	11	42	48	12		BICR
Update 12-30	D 11.3.1	GFP-based cellular system to screen for regulators of PUMA expression	11	21	30	18	21	BICR
Update 25-42	D 11.7	Novel regulators of PUMA expression from siRNA oligonucleotide screen	11	21	39	6	9	BICR
	D 11.8	Data on initial characterisation of respective gene products	11	27	48	6	9	BICR
Original	D 12.1	Cell systems with knockdown of p53	12	12	12		6	NK12
	D 12.2 (70)	Candidate synthetic lethal interactions with p53	12	12	30	6	12	NK12
Update 12-30	D 12.3	Candidate synthetic lethal genes with p53 from siRNA screens	12	30	30	6	12	NK12
Update 25-42	D 12.4	Validated synthetic lethal interactions	12	36	42	12	6	NK12
Original	D 13.1	Reporter cell lines that carry stably expressed p53 reporter constructs, with or without tet-repressor system	13	6	9	4	10	IEO2
	D 13.2	Reporter cell lines that carry stably expressed p53 reporter constructs and conditional alleles of NPM-ALK or MDM4	13	12	15	4	10	IEO2
Update 12-30	D 13.3	Novel genes which control p53 activity and putative targets for the identification of new leads for cancer intervention strategies.	13	30	in progress	7	9	IEO2

Third Annual Periodic Activity Report (2006)

	D 13.4	Biological assays for studying new pathways regulating p53 activity and new therapeutic approaches	13	30	in progress	7	9	IEO2
Update 25-42	D 13.3	Novel genes which control p53 activity and putative targets for the identification of new leads for cancer intervention strategies.	13	30	36	7	8	IEO2
	D 13.4	Biological assays for studying new pathways regulating p53 activity and new therapeutic approaches	13	30	in progress	7	7	IEO2
Original	D 14.1 (17)	Human and mouse reporter cell lines that stably express p16INK4a-GFP reporter construct.	14	6	18	6	4	NKI3
	D 14.2 (47)	Mouse reporter cell lines that stably express p16INK4a-GFP reporter construct under the control of the endogenous INK4a locus	14	12	18	12	2	NKI3
	D 14.3 (64)	Novel INK4A/pRB pathway-specific genes, which provide new leads for cancer intervention strategies.	14	15	27	12	6	NKI3
	D 14.4 (90)	Initial characterization of the respective gene products	14	18	36	6	6	NKI3
Update 25-42	D 14.5	Initial characterization of the respective gene products (INK4a screen)	14	48	48	6	6	NKI3
	D 14.6	Initial characterization of the respective gene products (Gli screen)	14	48	48	6	6	NKI3
Original	D15.1 (18)	Reporter cell lines that stably express CCNE1/CCNA2 reporter constructs	15	6	24	6	11	UKBH
	D15.2 (48)	Novel pRB/E2F pathway specific genes which provide new leads for cancer intervention	15	12	30	6	1	UKBH
	D15.3 (91)	Initial biological characterization of genes identified in the RNAi screens	15	18	30	6	3	UKBH
	D15.4(92)	Expression analysis in primary human tumours, and determination of gene copy number	15	12	36	6	6	UKBH
Update 12-30	D15.5	A high throughput live cell based screening system using E2F/pRB reporter genes	15	24	24	6	6	UKBH

Third Annual Periodic Activity Report (2006)

	D15.6	Generation of stable human cell lines containing an EZH2-GFP reporter	15	30	Awaiting outcome of CCNA2 screen	6	6	UKBH
	D15.7	Generation of EZH2-GFP reporter cell lines validated for high throughput screening compatibility	15	33	Awaiting outcome of CCNA2 screens	6	6	UKBH
Update 25-42	D15.8	Establishment and performance of HCS using complete NKI library	15	30	30	6	6	UKBH
Original	D16.1 (93)	Identify the pathways by which the Rb family of proteins controls telomere length	16	18	12	7,5	7,5	CNIO1
	D16.2 (94)	Identification of new activities important for telomere elongation	16	18	12	7,5	7,5	CNIO1
	D16.3 (95)	New mouse models and cellular systems	16	18	cancelled (alternative strategy)	7,5	7,5	CNIO1
Update 12-30	D16.4	Validation of reporter cell line for genetic screens aimed at the identification of genes that alter the structure of telomeric heterochromatin	16	15	15	5	5	CNIO1
	D16.5	Novel regulators of telomeric chromatin	16	24	48	6	6	CNIO1
	D16.6	Epigenetic regulators of telomere function in the context of loss of Rb function	16	30	48	12	12	CNIO1
Update 25-42	D16.7	Mechanisms by which the identified genes regulate telomere function	16	36	42	12	6	CNIO1
	D16.8	Implication of the identified genes in telomere-driven cellular processes relevant to cancer	16	42	48	12	0	CNIO1
Original	D 17.1	WI38 diploid fibroblast cells with stable knockdown of pRb expression.	17	24	Cancelled, alternative strategy	4	10	IEO
	D 17.3	Novel genes that are synthetic lethal with pRb-loss.	17	36	Cancelled, alternative strategy	4	10	IEO
Update 12-30	D 17.2	Primary cells with inducible expression of NPM-ALK	17	24	Cancelled, alternative strategy	6	3	IEO

Third Annual Periodic Activity Report (2006)

	D 17.4	Identification of genes whose expression allows the expression of NPM/ALK	17	36	Cancelled, alternative strategy	6	3	IEO
	D 17.5	NPM knock-out mouse	17	36	36		6	IEO
	D 17.6	Characterization of NPM cytoplasmic localization in AMLs	17	36	36		6	IEO
Update 25-42	D 17.7	CRE-Lox NPM mutant transgenic mouse model	17	42	In progress	6	3	IEO
	D 17.8	New genes involved in the checkpoint response (possibly) activated in stem cells by expression of the NPM mutant or genes whose inactivation show synthetic lethal interaction with the expression of the NPM mutant	17	48	Not yet started	5	-	IEO
	D 17.9	ES mouse cell line with inducible siRNA for NPM	17	36	In progress	4	3	IEO
	D 17.10	NPM role in DNA damage repair	17	42	In progress	4	3	IEO
	D 17.11	Two different NPM isoforms Inducible KO mice	17	48	In progress	5	3	IEO
	D 17.12	New genes responsible for the checkpoint activation induced by the lack of NPM	17	48	In progress	3	3	IEO
Original	D 18.1	Effect of Raf-1 conditional ablation in a number of mouse tumour models	18	12	12	12	12	GEM
	D 18.2	Effect of B-Raf conditional ablation in a number of mouse tumour models	18	12	12	12	12	GEM
	D 18.3	Identification of a putative novel effector of Raf-1	18	12	12	18	18	GEM
	D 18.4	Identification of a putative B-Raf downstream pathway relevant for tumourigenesis in the Rip-Tag model	18	18	18	18	18	GEM
Update 12-30	D 18.5	Structure/function information on the interaction between Raf-1 and its interacting kinase Rok- α	18	24	24-30	15	15	GEM
	D 18.6	Validated Raf-1 effectors in transformed keratinocytes in vitro	18	27	27	12	12	GEM

Third Annual Periodic Activity Report (2006)

	D 18.7	Validation of ERK phosphorylation/VEGF production as B-Raf downstream pathways relevant for pancreatic adenocarcinoma development in tumour derived β -cell lines	18	27	27	12	12	GEM
	D 18.8	Effect of Raf-1 knockdown in human epithelial cell lines	18	30	30	9	9	GEM
	D 18.9	Effect of B-Raf knockdown in human insulinoma cell lines	18	30	30	9	9	GEM
Update 25-42	D 18.10	Effect of Raf-1 ablation on EMT and metastasis	18	36	achieved and in progress	12	12	GEM
	D 18.11	Effect of B-Raf ablation on the development of epithelial tumours	18	42	achieved	9	9	GEM
	D 18.12	Effect of B-Raf ablation on the maintenance of epithelial tumours	18	42	to be started	9	9	GEM
	D 18.13	Epidermis-restricted double (Raf-1/B-Raf) KO	18	33	achieved	6	6	GEM
	D 18.14	Effect of Epidermis-restricted double (Raf-1/B-Raf) KO on development and maintenance of epithelial tumours	18	48	cancelled	12	12	GEM
	D 18.15	Effect of B-Raf ablation on the tumour stroma in the insulinoma model	18	36	achieved/in progress	6	6	GEM
	D 18.16	Status of tumorigenesis-relevant signalling pathway that cross-talk with, or are downstream of, Raf, by immunohistochemical analysis of in tumour sections and/or biochemically in tumour explants.	18	48	in progress	18	18	GEM
Original	D 19.1	Novel genes involved in premature senescence, which provide new leads for cancer intervention strategies	19		In progress	6	9	Pasteur
	D 19.2	Reporter cell lines that stably express PML reporter constructs	19		Cancelled	6	1	Pasteur
	D 19.3	Initial characterization of the respective gene products	19		To be started	6	1	Pasteur
	D 19.4	Expression analysis in primary human tumours	19		To be started	6	1	Pasteur

Third Annual Periodic Activity Report (2006)

Update 12-30	D 19.5	Mediators of PML-induced senescence	19	21	36 (in part)	6	6	Pasteur
	D 19.6	Mediators of PIASy-induced senescence	19	24	36 (in part)	6	6	Pasteur
	D 19.7	shRNA library targeting enzymes involved in SUMO and SUMO-like modifications	19	27	42	6	3	Pasteur
	D 19.8	Enzymes involved in SUMO and SUMO-like modifications relevant to PML- and PIASy-induced senescence	19	30	48	6	3	Pasteur
Update 25-42		Due to the delay in the realization of the tasks planned for months 1-30, no additional items have been included into this update for months 25-42						
Original	D 20.1 (22)	IMR90-derived cells representing the sequential steps in the transformation process	20	6	6	9	9	CNIO2
	D 20.2 (52)	List of candidate genes associated with each step in the transformation process	20	12	12	9	9	CNIO2
	D 20.3 (100)	Markers for OIS	20	18	12	9	9	CNIO2
	D 20.4 (101)	New mouse models of tumourigenesis	20	18	18	9	9	CNIO2
Update 12-30	D 20.5	shRNA vectors knocking down the expression of previously identified OIS	20	18	25	15	10	CNIO2
	D 20.6	Identification of OIS markers as mediators of senescence or neoplastic transformation	20	30	36	24	0	CNIO2
Update 25-42	D 20.7	Reporter cell system suitable for shRNA screen for E2F1 regulators	20	27	27	3	1	CNIO2
	D 20.8	Novel regulators of E2F1	20	45	45	12	0	CNIO2
	D 20.9	RNAi library against non-coding regions of the INK4a/ARF locus	20	30	30	6	1	CNIO2
	D 20.10	Mechanisms of RNAi-directed epigenetic silencing in the context of tumour suppression	20	48	48	15	0	CNIO2

Third Annual Periodic Activity Report (2006)

Original	D21.1	Novel Myc/Miz/Tgfb- and Myc/Miz1/APC- pathway-specific genes	21	12	12	12	12	UNIMAR
	D21.2	Initial characterization of the respective gene products and mode of action	21	15	15	3	3	UNIMAR
	D21.3	shRNAs that regulate Myc apoptotic functions	21	18	18	3	3	UNIMAR
	D21.4	Expression analysis in primary human tumors	21	18	30	3	0	UNIMAR
Update 12-30	D21.5	Biochemical Function of Usp28 (Manuscript)	21	12	36	9	9	UNIMAR
	D 21.6	Biochemical Function of Parp2/Rad1 (Manuscript)	21	18	36	12	9	UNIMAR
	D 21.7	Assay system for developmental control of Myc-induced apoptosis	21	24	24 (in part)	6	0	UNIMAR
Update 25-42	D21.8	Role of H2.a.z in gene regulation by Myc (Manuscript)	21	48	48	12	12	UNIMAR
Original	D22.1	Barcode Arrays for Myc function	22	21	21	12	12	UNIMAR
	D22.2	Data describing mode of action of Myc/Barcode hits	22	30	30	6	6	UNIMAR
	D22.3	Barcode Arrays for Myc function in mouse cells	22	30	42	6	0	UNIMAR
	D22.4	Biological characterization of Hits from Mouse Barcodes	22	30	48	6	0	UNIMAR
Update 12-30	D22.5	Characterization of AURKA (Manuscript)	22	36	42	12	18	UNIMAR
	D 22.7	96 well format assays	22	48	48	6	6	UNIMAR
Original	D23.1	HSCs of various genotypes	23	18	24	12	12	ISREC1
	D23.2	Viruses that can be used to overexpress c-MYC in HSCs	23	21	21	9	9	ISREC1

Third Annual Periodic Activity Report (2006)

Update 12-30	D23.3	GFP expressing HSCs carrying the various mutant alleles	23	30	40	12	22	ISREC1
	D23.4	Data regarding the role of c-MYC, PTEN on HSC self-renewal	23	18	36	18	18	ISREC1
Update 25-42	D23.5	shRNA vectors that promote c-Myc ⁺ Pten ^{Δ/Δ} HSC self-renewal or survival	23	42	42	18	0	ISREC1
	D23.6	Pten deficient leukemic stem cells	23	48	48	18	0	ISREC1
	D23.7	Inducible lentiviral system to overexpress or knock down genes in hematopoietic stem cells in vitro and in vivo	23	48	48	18	18	ISREC1
	D23.8	Data showing that Pten controls HSC mobilization and prevents T cell leukemias	23	48	48	18	18	ISREC1
Original	D 24.1	Candidate gene-drug interactions with cancer drugs	24	20	20/20	12	12	NK12
Update 12-30	D. 24.2	Genes that modulate sensitivity to Herceptin	24	36	36/36	6	6	NK12
	D 24.3	Genes that modulate sensitivity to Iressa	24	36	36/36	6	6	
Update 25-42	D 24.4	Validated gene-drug interactions	24	36	36/36	6	6	NK12
	D 24.5	Molecular basis of gene drug interactions	24	48		6	-	NK12
Original	D 25.1	Lentiviral reporter constructs to generate transgenic p53 and E2F effector reporter mice	25	6	Revised	6	6	MDC
	D 25.2	A large number of individual primary lymphomas generated as matched pairs (i.e. senescence-competent versus -defective as well as drug-sensitive versus drug-resistant)	25	6	6	24	24	MDC
	D 25.3	A versatile test system to validate the impact of candidate genes that were found in other screens (synthetic lethality screen, ERMtag screen) on drug resistance in vivo	25	12	12	6	6	MDC

Third Annual Periodic Activity Report (2006)

Update 12-30	D 25.4	Loci and candidate genes identified by Splinkerette-PCR and subsequent sequencing from in vitro senescence escape screens	25	18 and 24	18 and 24, on hold	18	18	MDC
	D 25.5	Loci and candidate genes identified by Splinkerette-PCR and subsequent sequencing from in vivo senescence escape screens	25	24	36, on hold	18	18	MDC
	D 25.6	Differential gene expression profiles (Affymetrix) comparing senescence-competent and -resistant clones in vitro	25	27	48 (revised)	6	6	MDC
	D 25.7	Differential gene expression profiles (Affymetrix) comparing senescence-competent and -resistant clones in vivo	25	30	48 (revised)	6	6	MDC
	D 25.8	Novel markers/assays to characterize the senescent conditions of cells/tissues	25	30	30	6	6	MDC
	D 25.9	Retroviral constructs that co-express RFP as reporter in vitro and in vivo	25	30	45 (revised)	6	6	MDC
	D 25.10	Lentiviral reporter constructs to generate transgenic p53 and E2F effector reporter mice	25	36	36 (revised)	12	12	MDC
Update 25-42	D 25.11	Lymphoma cells with stable long-term expression of dsRed-Tomato	25	42	42	6	6	MDC
	D 25.12	A Suv39h1-proficient/-deficient matched pair mouse lymphoma model	25	42	42	6	6	MDC
Original	D 26.1 (75)	Validated reporter mice to monitor tumour growth and tumour regression (tumour mass reporter)	26	9	12	8	8	NKI1
	D 26.2 (76)	Compound mutant mice of genotype: Rbflox/flox;POMC-Cre;POMC-luciferase	26	12	12	4	4	NKI1
	D 26.3 (77)	Compound mutant mice of genotype: p53flox/flox;Rbflox/flox;POMC-Cre;POMC-luciferase	26	12	16	4	3	NKI1
Update 12-30	D 26.4	Mesothelioma cell lines	26	18	21	4	4	NKI1
	D 26.5	Contribution of p53 and Ink4a/p19Arf to chemotherapy resistance of a non-lymphoid tumour	26	24	24	4	2	NKI1
Original	D 27.1 (30)	First prognostic gene expression signatures for one of the three types of human cancer	27	18	18/18	9	9	Agendia
	D 27.2 (38)	Second prognostic gene expression signatures for one of the three types of human cancer	27	18	18/18	9	9	Agendia

Third Annual Periodic Activity Report (2006)

	D 27.3 (61)	Third prognostic gene expression signatures for one of the three types of human cancer	27	18	15/18	9	9	Agendia
	D 27.4	Database of gene expression signatures from public domain microarray data sets	27	18	18/18	6	6	Agendia
Update 12-30	D. 27.5	Prognostic gene expression signature for lung cancer	27	36	36/36	6	6	Agendia
	D 27.6	Extended database of gene expression signatures from public domain microarray data sets	27	36	36/42	6	6	Agendia
	D 27.7	Customize Resolver software and hardware for storing database of microarray data	27	36	36/42	6	6	Agendia
Original	30.1	Recruitment of qualified personnel to the EC-funded positions	30	1-12 and 13-36 if appropriate at this stage	1-12 and 13-36 if appropriate at this stage	2	2	GEM
	30.2	Organization of the annual Symposia	30	24, 36	24, 36	2	2	GEM
	30.3	Organization of workshops/Working groups	30	12, 24, 36 15 (IMT-NKI2) 17 (Pasteur-NKI2)	12, 24, 36	2	2	GEM
	30.4	Marie-Curie RTN application (first stage)	30	21	21	8	8	GEM
	30.5	Marie-Curie RTN application (second stage)	30	25	25	8	8	GEM
Original	D 31.1	INTACT Website (maintained throughout the lifetime of the Consortium and beyond)	31	6	6	9	9	UNIMAR
Update 25-42	D 32.1	Compounds that inhibit the proliferation of wild type and p53 null isogenic HCT-116 colon carcinoma cell lines	32	12	48	36	48.7	Vichem

List of milestones (months 1 – 42)

	No.	Milestone name	WP	Date due	Actual/Forecast delivery date	Lead contractor
Original	M 4.1	Concentrated high titer viral supernatant	4	12	Achieved	IEO
	M 4.2	Standardized protocols to spot viruses on glass slides	4	18	Achieved	IEO
	M 4.3	Protocols for efficient immobilization of viral particles on glass slides.	4	18	Achieved	IEO
	M 4.4	Retroviral microarrays of primary cells	4	18	Achieved/In progress	IEO
Update 13-30	M 4.5	Concentrated high titer viral supernatant from RNAi kinase library	4	18	Delayed	IEO
	M 4.6	Retroviral array on chip of the kinase library	4	24	Delayed	IEO
	M 4.7	Normalized retroviral array on chip of RNAi kinase library	4	27	Not started yet	IEO
	M 4.8	Validation of efficiency of RNAi of reverse infection in array format of RNAi kinase library in human primary and cancer cells	4	30	Delayed	IEO
Update 25-42	M4.9	Optimized ns-TiO ₂ substrate for viral arrays	4	36	Achieved	IEO
	M 4.10	Array of pRetrosuper vectors of kinase library with analysis of RNA interference efficiency on chip	4	48	Not started yet	IEO
	M 4.11	Set up of an immunofluorescence assay on chip for detection of protein expression and localization in cells upon RNAi	4	36	achieved	IEO
Original	M 05.1	Generation of HIV-based vectors suitable for transgenesis of inducible RNAi.	05	21	In progress	NKI1
	M 05.2	Validated protocols for infection of preimplantation embryos	05	21	Achieved	NKI1
	M 05.3	Tet-repressor transgenic mouse lines	05	30	In progress	NKI1
Update 12-30	M 05.4	Validated p53 reporter lines	05	42	In progress	NKI1
Original	M 6.1	Validated siRNAs against the relevant components of the Wnt pathway	6	18	in progress	ISREC2
	M 6.2	Grafting conditions for LS174T cell line established	6	21	in progress	ISREC2
	M 6.3	Effect on tumour xenografts after knock-down of different signaling components evaluated	6	24	in progress	ISREC2
	M 6.4	Introduction and evaluation of single vector lentiviral construct for inducible expression of siRNA	6	18	in progress	ISREC2
	M 6.5	Different strategies to engraft primary tumour material assessed	6	30	not started yet	ISREC2
Update 12-30	M 6.1	Validated siRNAs against the relevant components of the Wnt pathway	6	18	Partially	ISREC2
	M 6.2	Grafting conditions for LS174T cell line established	6	21	Achieved	ISREC2
	M 6.3	Effect on tumour xenografts after knock-down of different signaling components evaluated	6	24	Achieved	ISREC2
	M 6.4	Introduction and evaluation of single vector	6	18	In progress	ISREC2

Third Annual Periodic Activity Report (2006)

		lentiviral construct for inducible expression of siRNA				
	M 6.5	Different strategies to engraft primary tumour material assessed	6	30	In progress	ISREC2
Update 25-42	M 6.1	Validated siRNAs against the relevant components of the Wnt pathway	6	18	Partially	ISREC2
	M 6.2	Introduction and evaluation of single vector lentiviral construct for inducible expression of siRNA	6	18	Achieved	ISREC2
	M 6.3	Effect on tumour xenografts after knock-down of different signaling components evaluated	6	24	Achieved	ISREC2
	M 6.4	Introduction and evaluation of single vector lentiviral construct for inducible expression of siRNA	6	18	Achieved	ISREC2
	M 6.5	Different strategies to engraft primary tumour material assessed	6	30	In progress	ISREC2
Original	M 7.1	Generation of reporter cell lines	7	6	Achieved	IEO1
	M 7.2	Performance of ERMtag screens	7	12	Cancelled (alt. strategy)	IEO1
	M 7.3	Performance of RNAi screens	7	18	Achieved	IEO1
	M 7.4	Initial biological characterization of genes identified in the (ERMtag, now RNAi) screen	7	18	In progress	UKBH
	M 7.5	Determination of expression of the first novel genes in primary human tumours	7	18	Achieved and in progress	UKBH
Update 12-30	M 7.6	Establishment of a reliable screening system for genes involved in the G2/M DNA damage checkpoint	7	12	Achieved	UKBH
	M 7.7	Performance of a second round of shRNA screens	7	18	Achieved	UKBH
	M 7.8	Candidate genes required for the G2/M checkpoint	7	18	Achieved	UKBH
	M 7.9	Validated mediators for the G2/M checkpoint	7	24	Achieved and in progress	UKBH
	M 7.10	Data concerning the function of the validated genes and their expression in cancer	7	30	In progress	UKBH
Update 25-42	M 7.11	Functional description of the validated genes and their implication in cancer	7	48	In progress and achieved	UKBH
	M 7.12	Establishment and performance of HCS	7	36	Not started yet	UKBH
Original	M 8.1	Generation of double knockout mice	8	6	6	CNIO1
	M 8.2	Generation of RNAi retroviral vectors for telomerase and telomere binding proteins	8	12	6	CNIO1
	M 8.3	Performance of gene array analysis	8	18	12	CNIO1
	M 8.4	Validation of gene array results	8	18	18	CNIO1

Third Annual Periodic Activity Report (2006)

	M 8.5	Screening of the different mouse models and cellular systems using high-titer RNAi libraries for identification of additional targets	8	18	Modified and replaced by M 8.8 due to change of strategy	CNIO1
Update 12-30	M 8.6	Generation of a human cells expressing dominant negative hTRF2 (DNhTRF2)	8	15	15	CNIO1
	M 8.7	Set-up and validation of the screening system	8	18	Partially achieved	CNIO1
	M 8.8	shRNA screen and isolation of shRNA inserts bypassing cell death induced by telomere dysfunction	8	24	cancelled	CNIO1
	M 8.9	Validation and characterization of candidate genes as mediators of telomere damage-driven apoptosis	8	27	cancelled	CNIO1
	M 8.10	Characterization of clones that bypass telomere-driven cell death for their ability to regulate telomere length	8	30	cancelled	CNIO1
Original	D 9.1 (84)	Identification of new targets for tumour progression and metastatic disease	9	18	24	10
	D 9.2 (12)	New RNAi retroviral vectors	9	6	24	10
	D 9.3 (85)	New mouse models	9	18	18	10
Update 12-30	D 9.4	High-throughput Q-FISH telomere length determination method	9	15	18	3
	D 9.5	Telomerase reporter cell line	9	15	15	1
	D 9.6	Genes that regulate telomere length and/or telomerase activity in a human tumour cell line	9	24	42	5
	D 9.7	Identification of novel regulators of telomere length and/or telomerase activity with relevance to telomere-driven tumourigenesis and metastasis in vivo	9	30	48	6
Original	M 10.1	Generation of GFP/RFP reporter cell lines	10	12	Achieved	BICR
	M 10.2	Characterisation of GFP/RFP reporter cell lines to assess suitability for screens	10	15	Achieved	BICR
	M 10.3	Performance of RNAi screens	10	21	Not started yet	BICR
	M 10.4	Performance of ERMtag screens	10	21	Cancelled	BICR
	M 10.5	Initial biological characterization of genes identified in the (ERMtag, now RNAi) screen	10	24	Not started yet	BICR
	M 10.6	Validation of expression of the first novel genes in tissue culture	10	24	Not started yet	BICR
Update 12-30	M 10.7	Development of p21-renilla and Bax-firefly luciferase systems for use as a screen	10	18	Achieved	BICR
	M 10.8	Development of PG13-renilla and Bax-firefly luciferase systems for use as a screen	10	33	Achieved and in progress	BICR

Third Annual Periodic Activity Report (2006)

	M 10.8.2	Generation of reporter cell lines expressing PG13-renilla and Bax-firefly luciferase for use as a screen	10	30	Not started yet	BICR
Update 25-42	M 10.8.2	Generation of reporter cell lines expressing PG13-renilla and Bax-firefly luciferase for use as a screen	10	30	Achieved	BICR
	M 10.5	Initial biological characterization of genes identified in the (ERMtag, now RNAi) screen	10	21	Achieved and in progress	BICR
	M 10.6	Validation of expression of the first novel genes in tissue culture	10	21	In progress	BICR
Original	M11.1	Generation of reporter cell lines	11	15	Achieved	BICR
	M 11.2	Characterization of cell lines	11	18	Achieved	BICR
	M 11.3	Performance of RNAi screens	11	21	Achieved	BICR
	M 11.4	Initial biological characterization of genes identified in the (ERMtag, now RNAi) screen	11	27	Not started yet	BICR
	M 11.5	Comparison of identified genes with genes obtained from other workpackages	11	36	Not started yet	BICR
	M11.6	Analysis of expression of identified genes in primary tumours by database analysis and screening of multiple tissue arrays by ISH.	11	36	Not started yet	BICR
	M 11.7	Validation of identified genes in tissue culture	11	42	Not started yet	BICR
Update 12-30	M 11.2.1	Development of GFP reporter assay for use as a screen	11	21	Achieved	BICR
Update 25-42	M 11.8	Performance of screen using siRNA oligonucleotide library	11	36	Achieved	BICR
	M 11.9	Initial biological characterization of genes identified in M 11.8	11	48	In progress	BICR
	M 11.10	Validation of genes identified in M11.8 in tissue culture	11	48	In progress	BICR
Original	M 12.1	Isogenic cell lines with and without expression of p53	12	12	achieved	NKI2
	M 12.2	Performance of synthetic lethal screens	12	24	in progress	NKI2
Update 12-30	M 12.3	Candidate synthetic lethal genes with p53 from siRNA screens	12	30	in progress	NKI2

Third Annual Periodic Activity Report (2006)

Update 25-42	M 12.4	Validated synthetic lethal hits with p53	12	42	in progress	NKI2
Original	M 13.1	Generation of U2OS-GFP and U2OS-GFP+tet-repressor cell lines	13	6	achieved	IEO2
	M 13.2	Generation of the above cell lines with inducible expression of NPM-ALK and MDM4	13	12	achieved-not useful	IEO2
	M 13.3	RNAi and ERMTAG screenings and identification of relevant genes	13	36	cancelled alternative strategy	IEO2
Update 12-30	M 13.4	Validation of relevant genes coming from the screenings	13	30	in progress (modified strategy)	IEO2
	M 13.5	Identification of new pathway regulating p53 activity and possible new therapeutic targets	13	30	in progress	IEO2
Update 25-42	M 13.4	Validation of relevant genes (new strategy)	13	30	achieved	IEO2
	M 13.5	Identification of new pathway regulating p53 activity and possible new therapeutic targets (new strategy)	13	30	in progress	IEO2
Original	M 14.1	Generation of reporter cell lines	14	6	Achieved 18	NKI3
	M 14.2	Performance of ERMtag screens	14	12	On hold until proven feasible with GLI (see WP3)	NKI3
	M 14.3	Performance of RNAi screens	14	12	In progress 27	NKI3
	M 14.4	Initial biological characterization of genes identified in the (ERMtag, now RNAi) screen	14	18	Not started yet	NKI3
	M 14.5	Determination of expression of the first novel genes in primary human tumors	14	18	Not started yet	NKI3
Update 12-30	M 14.6	Establishment of suitable lentiviral reporter system for regulators of p16INK4a	14	12	Achieved 12	NKI3
	M 14.7	Testing and performance of shRNA screens for INK4a regulators (pooled format and/or single well based assays).	14	18	In progress 36	NKI3
	M 14.8	Final assessment of feasibility to use ERM tag screens.	14	24	Achieved and in progress 27	NKI3
	M 14.9	Isolation of genes targeted by shRNAs from the screens.	14	30	In progress 39	NKI3
	M 14.10	If feasible, performance of ERM tag screens with p16INK4a-reporters in tet-off cell lines.	14	30	On hold until proven feasible with GLI (see WP3)	NKI3

Third Annual Periodic Activity Report (2006)

Update 25-42	M 14.11	Validation and initial characterization of INK4a regulators.	14	30	Not started yet	NKI3
	M 14.12	Multimerized p16INK4a-LUC reporter	14	36	Not started yet	NKI3
	M 14.13	Performance of expanded Gli ERMtag screen	14	39	In progress	NKI3
	M 14.14	Performance of p16INK4a-LUC siRNA screen	14	42	Not started yet	NKI3
	M 14.15	Candidate genes involved in Gli regulation	14	48	In progress	NKI3
Original	M15.1	Generation of reporter cell lines	15	6	Achieved	UKBH
	M15.2	Performance of ERMtag screens	15	12	Cancelled (alt. strategy)	UKBH
	M15.3	Performance of RNAi screens	15	18	Awaiting 2 nd generation arrayed shRNA library	UKBH
	M15.4	Initial biological characterization of genes identified in the (ERMtag, now RNAi) screen	15	18	In progress	UKBH
	M15.5	Determination of expression of the first novel genes in primary human tumours	15	18	Achieved and in progress	UKBH
Update 12-30	M15.6	Development of a system for automated screening of the human shRNA library	15	18	Achieved	UKBH
	M15.7	Generation of CCNA2 reporter cell lines validated for high throughput screening compatibility	15	24	Achieved	UKBH
	M15.8	Generation of stable human cell lines containing an EZH2-GFP reporter	15	30	Awaiting outcome of CCNA2 screens	UKBH
	M15.9	Generation of EZH2-GFP reporter cell lines validated for high throughput screening compatibility	15	24	Awaiting outcome of CCNA2 screens	UKBH
Update 25-42	M15.10	Functional description and validation of genes identified in the kinome sub screen and their implication in cancer	15	42	In progress	UKBH
	M15.11	Establishment and performance of HCS using entire NKI arrayed library	15	48	Not started yet	UKBH
Original	M16.1	Generation of RNAi retroviral vectors against known telomere elongating activities	16	6	12	CNIO1
	M16.2	Study the functional interactions between the Rb family of proteins and other activities involved in telomere maintenance	16	12	12	CNIO1
	M16.3	Gene array analysis in the different systems to identify new activities important for telomere length regulation	16	18	Cancelled, alternative strategy	CNIO1
	M16.4	Validation of the genes obtained using gene arrays by "real-type" RT-PCR and using RNAi vectors	16	18	Cancelled, alternative strategy	CNIO1

Third Annual Periodic Activity Report (2006)

	M16.5	Screening of the different mouse models and cellular systems using high-titer RNAi libraries for identification of additional targets	16	18	Cancelled, alternative strategy	CNIO1
Update 12-30	M16.6	Validation of the screening system for the identification of genes that alter the structure of telomeric heterochromatin	16	15	15	CNIO1
	M16.7	shRNA screen and isolation of shRNA inserts that alter the structure of telomeric heterochromatin	16	24	24	CNIO1
	M16.8	Validation and characterization of candidate genes with respect to their effects on telomeric heterochromatin and telomere length	16	30	42	CNIO1
	M16.9	Characterization of identified genes as epigenetic regulators of telomere function in the context of loss of Rb function	16	36	48	CNIO1
Update 25-42	M 16.10	Role of candidate genes in telomere-driven cellular processes relevant to cancer	16	42	48	CNIO1
Original	M 17.1	Generation of Rb inactive WI38 and primary cells with inducible NPM/ALK.	17	24	Cancelled, Alternative strategy	IEO
	M 17.2	Screening of novel genes with RNAi library	17	36	Cancelled, Alternative strategy	IEO
Update 12-30	M 17.1	Generation of primary cells with inducible NPM/ALK	17	24	Cancelled, Alternative strategy	IEO
	M 17.2	Screening of novel genes whose downregulation allows NPM/ALK expression	17	36	Cancelled, Alternative strategy	IEO
	M 17.3	Generation of NPM knock-out mouse	17	36	Achieved	IEO
	M 17.4	Role of NPM cytoplasmic localization in AMLs	17	36	Achieved	IEO
Update 25-42	M 17.5	Generation of a transgenic CRE-Lox NPM mutant mouse model	17	42	In progress	IEO
	M 17.6	Screens of genes involved in the checkpoint response (possibly) activated in stem cells by expression of the NPM mutant or genes whose inactivation show synthetic lethal interaction with the expression of the NPM mutant by RNAi library approach	17	48	Not yet started	IEO
	M 17.7	Generation of an inducible NPM RNAi ES mouse cell line	17	36	In progress	IEO
	M 17.8	NPM role in DNA damage repair	17	42	In progress	IEO
	M 17.9	Generation of inducible KO mice for the two different NPM isoforms	17	48	In progress	IEO
	M 17.10	Screening of genes that are likely to be responsible for the checkpoint activation induced by the lack of NPM by RNAi library approach	17	48	Not yet started	IEO
Original	M 18.1	Effect of Raf-1 conditional ablation in a number of mouse tumour models (achieved)	18	12	Achieved	GEM
	M 18.2	Effect of B-Raf conditional ablation in a number of mouse tumour models	18	12	Achieved	GEM
	M 18.3	Identification of a putative novel effector of Raf-1	18	12	Achieved	GEM

Third Annual Periodic Activity Report (2006)

	M 18.4	Identification of a putative B-Raf downstream pathway relevant for tumourigenesis in the Rip-Tag model	18	18	Achieved	GEM
Update 12-30	M 18.5	Structure/function information on the interaction between Raf-1 and its interacting kinase Rok- α	18	24	Achieved and in progress	GEM
	M 18.6	Validated Raf-1 effectors in transformed keratinocytes in vitro	18	27	Achieved and In progress	GEM
	M 18.7	Validated B-Raf-1 effectors in vitro in tumour derived β -cell lines	18	27	in progress	GEM
	M 18.8	Initial comparison between the results obtained by conditional Raf-1 ablation in the in vivo and in vitro system in the mouse and those obtained in vitro by knockdown in human cancer cell lines	18	30	In progress	GEM
	M 18.9	Initial comparison between the results obtained by conditional B-Raf ablation in the in vivo and in vitro system in the mouse and those obtained in vitro by knockdown in human cancer cell lines	18	30	In progress	GEM
	M 18.10	Towards the validation of Rok- α as an effector in vivo	18	30	In progress	GEM
Update 25-42	M 18.11	Effect of Raf-1 ablation on EMT and metastasis	18	36	Achieved and In progress	GEM
	M 18.12	Effect of B-Raf ablation on the development of epithelial tumours	18	42	Achieved and In progress	GEM
	M 18.13	Effect of B-Raf ablation on the maintenance of epithelial tumours	18	42	In progress	GEM
	M 18.14	Effect of an epidermis-restricted double (Raf-1/B-Raf) KO	18	33	Achieved	GEM
	M 18.15	Effect of Epidermis-restricted double (Raf-1/B-Raf) KO on development and maintenance of epithelial tumours	18	48	Not achievable/cancelled	GEM
	M 18.16	Effect of B-Raf ablation on the tumour stroma in the insulinoma model	18	36	Achieved and In progress	GEM
	M 18.17	Status of tumourigenesis-relevant signalling pathway that cross-talk with, or are downstream of, Raf, by immunohistochemical analysis of in tumour sections and/or biochemically in tumour explants.	18	48	In progress	GEM
Original	M 19.1	Generation of reporter cell lines	19	6	Cancelled	Pasteur
	M 19.2	Performance of ERMtag screens	19	12	Cancelled	Pasteur
	M 19.3	Performance of RNAi screens on PML-expressing human fibroblasts and reporter cell lines	19	15	Achieved and in progress	Pasteur
	M 19.4	Initial biological characterization of genes identified in the ERMtag screen	19	18	Cancelled	Pasteur
	M 19.5	Determination of expression of the first novel genes in primary human tumours	19	18	To be started	Pasteur
Update 12-30	M 19.6	shRNA screen bypassing PML-induced senescence	19	21	Achieved and in progress	Pasteur
	M 19.7	shRNA screen bypassing PIASy-induced senescence	19	24	On hold	Pasteur
	M 19.8	SUMO shRNA sub-library screen bypassing PML- and PIASy-induced senescence	19	27	Not started yet	Pasteur
	M 19.9	Characterization of genes involved in PML/PIASy/SUMO-dependent senescence	19	30	Achieved and in progress	Pasteur
Update 25-42		Due to the delay in the realization of the tasks planned for months 1-30, no additional items have been included into this update for months 25-42				

Third Annual Periodic Activity Report (2006)

Original	M 20.1	Generation and characterization of cell lines	20	6	Achieved	CNIO2
	M 20.2	Microarray hybridization and analysis of data	20	9	Achieved	CNIO2
	M 20.3	Validation and further characterization of candidate genes	20	12	Achieved	CNIO2
	M 20.4	Use of OIS markers in animal models of oncogene induction	20	18	Achieved	CNIO2
	M 20.5	Generation of new mouse models of tumourigenesis	20	18	Achieved	CNIO2
Update 12-30	M 20.6	Knock-down of OIS in a model system of stepwise oncogenic transformation	20	18	In progress	CNIO2
	M 20.7	Biological validation of OIS markers as mediators of senescence or neoplastic transformation	20	30	To be started	CNIO2
Update 25-42	M 20.8	shRNA screen for novel regulators of E2F1	20	33	Terminated	CNIO2
	M 20.9	Functional studies of novel regulators of E2F	20	45	Terminated Cancelled	CNIO2
	M 20.10	RNAi screen of non-coding regions of the INK4a/ARF locus for domains inducing epigenetic silencing	20	36	In progress	CNIO2
	M 20.11	Mechanistic studies on RNAi-induced epigenetic silencing of the tumour suppressor locus INK4a/ARF	20	48	To be started	CNIO2
Original	M 21.1	Completion of Miz1 RNAi screens	21	12	Achieved	UNIMAR
	M 21.2	Completion of Myc RNAi screens	21	15	Achieved	UNIMAR
	M 21.3	Initial biological characterization of genes identified in the Miz1 RNAi screens and their mode of action.	21	18	Achieved	UNIMAR
	M 21.4	Determination of expression of the first novel genes in primary human tumours	21	21	Achieved	UNIMAR
	M 21.5	Initial biological characterization of genes identified in the Myc RNAi screen and their mode of action	21	24	Achieved	UNIMAR
Update 12-30	M 21.6	Biochemical Mode of Action of one of MycRNAi screen genes understood	21	30	Achieved	UNIMAR
	M 21.7	Biochemical Mode of Action of one of Miz1 RNAi screen genes understood	21	36	Achieved	UNIMAR
	M 21.8	Novel assay systems to analyze developmental control of Myc-induced apoptosis	21	42	Not started yet	UNIMAR
	M 21.9	Validated mediators of Myc transcriptional activation	21	48	In progress/48	UNIMAR
Update 25-42	M21.10	Inducible Knockdown of Usp28 in Xenograft Model	21	48	In progress/48	UNIMAR and ISREC1
Summary	M 22.1	Completion of initial RNAi barcode screens	22	21	21	UNIMAR

Third Annual Periodic Activity Report (2006)

	M 22.2	Initial characterization of Miz1 hits that are obtained	22	24	Delayed until sequence-validated library is available	UNIMAR
	M 22.3	Characterization of Myc hits that are obtained	22	30	Achieved	UNIMAR
	M 22.4	Mouse RNAi barcode screens.	22	30	Delayed until sequence-validated library is available	UNIMAR
	M 22.5	Biological characterization of Hits from Mouse Barcodes	22	30	Delayed until sequence-validated library is available	UNIMAR
Update 12-30	M 22.6	Completion of Candidate Screen in Neuroblastoma Cells	22	30	Completed	UNIMAR
	M 22.7	Biochemical Mode of Action of one of RNAi screen genes understood	22	36	In progress/42	UNIMAR
	M 22.8	Novel assay systems to analyze synthetic lethal interactions	22	48	In progress/48	UNIMAR
Update 25-42	M22.9	Identification of one shRNA with synthetic lethal effects in U2OS-MycER identified	22	48	In progress/48	UNIMAR
Original	M 23.1	Establishment of LTC-IC assays	23	18	Achieved	ISREC1
	M 23.2	Establishment and production of viral vectors that can be used to overexpress c-MYC	23	21	Achieved	ISREC1
Update 12-30	M 23.3	Establishment of the time points each genotype lost HSC self-renewal activity	23	30	Achieved	ISREC1
	M 23.4	Mutant mice (Pten; p19ARF, MYC) carrying the Sca-1::GFP allele	23	30	Not achieved, change of strategy	ISREC1
Update 25-42	M 23.5	Identification of genes that promote c-Myc ⁺ Pten ^{ΔΔ} HSC self-renewal or survival	23	48	Not started yet	ISREC1
	M 23.6	Identification and molecular characterization of Pten deficient leukemic stem cells	23	48	Achieved, second part cancelled	ISREC1
	M23.7	Inducible lentiviral system to overexpress or knock down genes in hematopoietic stem cells in vitro and in vivo	23	48	Achieved	ISREC1
	M23.8	Data showing that Pten controls HSC mobilization and prevents T cell leukemias	23	48	Achieved and in progress	ISREC1
Original	M 24.1	Candidate genetic interactions with cancer drugs	24	20	Mostly Achieved	NKI2
Update 12-30	M 24.3	Candidate genes that show genetic interaction with additional cancer drugs	24	36	36/36	NKI2
Update 25-42	M 24.4	Validated gene-drug interactions	24	42	In progress	NKI2
	M24.5	Understanding of molecular basis for gene-drug interaction	24	48	started	NKI2

Third Annual Periodic Activity Report (2006)

Original	M 25.1	Generation of lentiviral reporter constructs	25	6	In progress/ Strategy revised	MDC
	M25.2	Availability of a two-colour fluorescence imaging system to monitor tumour progression and treatment responses in lymphoma-bearing mice	25	6	(Partly) Achieved	MDC
	M25.3	Isolation of numerous (i.e. > 20) primary myc/bcl2 double-transgenic lymphomas	25	6	Achieved	MDC
	M25.4	Generation of senescence-escape clones in clonogenic assays	25	12	Achieved	MDC
Update 12-30	M 25.5	Generation of numerous matched pairs of senescence-competent and –resistant myc/bcl2 lymphomas in vivo	25	18	Achieved and ongoing	MDC
	M25.6	Identification of candidate loci from insertional mutagenesis in vitro screens	25	18	Achieved and on hold (revised)	MDC
	M25.7	Generation of human B cell lymphoma cell lines as a test system for an siRNA-library based screen	25	21	Achieved	MDC
	M25.8	Identification of candidate loci from insertional mutagenesis in vivo screens	25	24	In progress and on hold (revised)	MDC
	M25.9	Expression profiles from in vitro-senescence screens	25	27	Not yet started	MDC
	M25.10	Expression profiles from in vivo-senescence screens	25	30	Not yet started	MDC
	M25.11	Novel senescence biomarker/bioassays	25	30	Achieved	MDC
	M25.12	Availability of a two-colour fluorescence imaging system to monitor tumour progression and treatment responses in lymphoma-bearing mice	25	36	In progress/mostly achieved	MDC
Update 25-42	M25.13	Assessment of the contribution of Suv39h1 defects on drug resistance in a mouse lymphoma model in vivo	25	24	In progress	MDC
	M25.14	Identification of an epigenetic drug-inducible senescence signature in human lymphoma cells	25	21	In progress	MDC
Original	M 26.1	Tumour mass reporters are validated and ready for distribution	26	12	Achieved	NKI1
	M 26.2	Resistance pattern of the 3 compound mutant mouse lines, predisposed to pituitary tumours, have been determined	26	12	Cancelled (alt. strategy)	NKI1
	M 26.3	Compound mutant mice developing pituitary tumors available for intervention studies	26	18	Achieved	NKI1
Update 12-30	M 26.4	Set of mesothelioma cell lines established	26	21	Achieved	NKI1
	M 26.5	Compare growth characteristics and chemotherapy resistance of Nf2;p53 and Nf2;lnk4a/p19Arf mutant cell lines	26	30	36	NKI1
	M 26.6	Perform CGH and expression array analysis to search for new targets for intervention.	26	30	36	NKI1
	M 26.7	Show effects of specific inhibition of genes found in task 6 on growth of cells in vitro and in vivo.	26	33	42	NKI1
Original	M27.1	First prognostic gene expression signatures for one of the three types of human cancer	27	18	Completed	Agendia
	M27.2	Second prognostic gene expression signatures for one of the three types of human cancer	27	18	Completed	Agendia
	M27.3	Third prognostic gene expression signatures for one of the three types of human cancer	27	18	Completed	Agendia
	M27.4	Database of gene expression signatures from public domain microarray data sets	27	18	completed	Agendia
date 12-	M 27.5	Prognostic gene expression signature for lung cancer	27	36	In progress	Agendia

Third Annual Periodic Activity Report (2006)

	M27.6	Extended database of gene expression signatures from public domain microarray data sets	27		In progress	Agendia
	M27.7	Customize Resolver software and hardware for storing database of microarray data	27		In progress	Agendia
Update 25-42	M 27.5	Prognostic gene expression signature for lung cancer	27	36	Achieved	Agendia
	M27.6	Extended database of gene expression signatures from public domain microarray data sets	27	42	In progress	Agendia
	M27.7	Customize Resolver software and hardware for storing database of microarray data	27	42	In progress	Agendia
	M27.8	Validate lung cancer prognostic profile	27	48	started	Agendia
Original	M 30.1	Recruitment of qualified personnel to the funded positions	30	12-36	Achieved	GEM
	M 30.2	Organization of annual Symposia held in January 2006 and February 2007	30	24, 36	Achieved	GEM
	M 30.3	Organizing 2-3 workshops and intensive working groups	30	12, 24, 36; 15 and 17	Achieved	GEM
	M 30.4	Prepare Marie Curie RTN application (first stage)	30	21	Achieved	GEM
	M 30.5	Prepare Marie Curie RTN application (second stage)	18	25	Achieved	GEM
Original	M 31.1	Website open	31	6	6	UNIMAR
Update 25-42	M 32.1	Installation of cellular inhibitory assay	32	36	Achieved	Vichem
	M 32.1	Testing of Chemical Validation Library (CVL) compounds	32	48	In progress	Vichem

Table 5 Workpackages – Plan and Status Barchart

	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 4																	
T 4.1	Optimize protocols for high throughput production and storage of retroviral supernatant.																
T 4.2	Setting up conditions for viral supernatant concentration.																
T 4.3	Establish efficient methods for viral particles spotting with automatic arrayer.																
T 4.4	Establish methods for efficient immobilization of viral particles on glass slides.																
T 4.5	Optimize protocols for high efficiency of infection for primary cells.																
T 4.6	Produce high titre viral supernatant from the pRETROSUPER kinase library.																
T 4.7	Analysis of titre and normalization of the supernatants																
T 4.8	Production of normalized retrovirus chip of the kinase library																
T 4.9	Analysis of efficiency of RNAi through inverse infection on primary and cancer cells by immunofluorescence analysis of selected known kinases																
T 4.10	Optimization of substrate properties and patterning on glass slide for high efficiency of infection in primary and cancer cells																
T 4.11	Array of pRetrosuper vectors of kinase library with analysis of RNA interference efficiency on chip																
T 4.12	Set up of an immunofluorescence assay on chip for detection of protein expression and localization in cells upon RNAi																
	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 6	Tasks																
T 6.1	Design and validate functional siRNA's for Legless1 and 2, Pygopus1 and 2, TCF4 and β -catenin																
T 6.2	Select LS174T cell line constitutively expressing luciferase and containing an inducible siRNA directed against Legless, Pygopus, TCF4 and β -catenin																
T 6.3	Establish and optimize conditions for reproducible engraftment of this cell line in a nude or NOD/SCID mouse model system: compare different clones with regard to engraftment, histology, and efficacy of gene of interest induction within the tumour																
T 6.4	Introduce luciferase-based non-invasive imaging of human tumour progression in mice. Subject tumour grafts to histological assessment of cell proliferation, apoptosis and cell differentiation upon induction of the siRNA cassette																
T 6.5	Construction and evaluation of a single vector lentiviral system containing a TetR-regulated siRNA cassette, and expression cassettes for TetR and luciferase																
T 6.6	Evaluate different approaches for engraftment of primary tumour material																
T 6.1	Design and validate functional siRNA's for Legless1 and 2, Pygopus1 and 2, TCF4 and β -catenin																
T 6.2	Select LS174T cell line constitutively expressing luciferase and containing an inducible siRNA directed against Legless, Pygopus, TCF4 and β -catenin																
T 6.3	Establish and optimize conditions for reproducible engraftment of this cell line in a nude or NOD/SCID mouse model system: compare different clones with regard to engraftment, histology, and efficacy of gene of interest induction within the tumour																
T 6.4	Introduce luciferase-based non-invasive imaging of human tumour progression in mice. Subject tumour grafts to histological assessment of cell proliferation, apoptosis and cell differentiation upon induction of the siRNA cassette																
T 6.5	Construction and evaluation of a single vector lentiviral system containing a TetR-regulated siRNA cassette, and expression cassettes for TetR and luciferase																

Third Annual Periodic Activity Report (2006)

[illegible][illegible][illegible]

Third Annual Periodic Activity Report (2006)

[illegible][illegible]

Third Annual Periodic Activity Report (2006)

	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 11	Tasks																
T11.1	Generation of PUMA-promoter GFP reporter																
T11.2	Evaluating responsiveness of reporter																
T11.3	Generation of stable cell lines expressing PUMA-GFP reporter																
T11.4	Testing cell lines to assess suitability for use in screens																
T11.5	Screening of cell lines expressing PUMA-GFP reporter using high-titer RNAi libraries																
T 11.6	Initial biological characterization of isolated genes																
T 11.7	Comparison of identified genes with genes obtained from other workpackages																
T 11.8	Analysis of expression of identified genes in primary tumours by database analysis and screening of multiple tissue arrays by ISH.																
T 11.9	Validation of the importance of the identified genes in regulation of PUMA expression in tissue culture systems																
T 11.5.2	Screening of cell line expressing PUMA-GFP reporter using siRNA oligonucleotide library																
T 11.6	Initial biological characterization of genes identified in T 11.5.2																
T 11.9	Validation of the importance of genes identified in T11.5.2 in regulation of PUMA expression in tissue culture systems																
	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 12	Tasks																
T 12.1a	Generation of human MCF-7 cells with stable knockdown of p53																
T 12.1b	Generation of human U2-OS cells with stable knockdown of p53																
T 12.2	Perform bar coded RNAi screens to identify synthetic lethal interactions with p53 (cancelled)																
T 12.3	Perform bar coded RNAi screens to identify synthetic lethal interactions with p53 in the presence of genotoxic stress (cancelled)																
T 12.4	Identify candidate synthetic lethal interactions with p53																
T 12.5	Optimize transfection of siRNA sets in 96-well format																
T 12.6	Establishment of a reliable screening system for p53 synthetic lethal interactions in 96-well plates																
T 12.7	Perform high throughput siRNA screen in p53 +/- and p53-/- cells in 96-well format																
T 12.8	Identify candidate synthetic lethal genes with p53 from siRNA screens																
T 12.9	Validate synthetic lethal hits																

	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 13	Tasks																
T 13.1	Generation of human U2OS osteosarcoma cell line expressing a reporter gene (GFP) under the control of a p53 dependent promoter (pGL13): U2OS-GFP.																
T 13.2	Generation of U2OS-GFP cell lines with inducible expression of NPM-ALK or MDM4.																
T 13.3	Screening of genes whose down regulation reactivate p53, using the above-described cell lines infected with high titer RNAi libraries.																
T 13.4	Generation of a U2OS-GFP cell line stably expressing the tet-repressor.																
T 13.5	Generation of U2OS-GFP+tet-repressor cell lines with inducible expression of NPM-ALK or MDM4																
T 13.6	Screening of genes that reactivate p53 activity using the cell lines																

Third Annual Periodic Activity Report (2006)

[illegible][illegible][illegible]

Third Annual Periodic Activity Report (2006)

[illegible][illegible][illegible]

Third Annual Periodic Activity Report (2006)

[illegible][illegible]

Third Annual Periodic Activity Report (2006)

[illegible][illegible][illegible]

Third Annual Periodic Activity Report (2006)

[illegible][illegible]

Third Annual Periodic Activity Report (2006)

	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 22	Tasks																
T22.1	Isolate RNA and cDNA from infection of U2OS-MycER cells with entire NKI library																
T22.2	Perform barcode analysis in Amsterdam together with NKI																
T22.3	Characterize positive shRNAs ("Outliers") from barcode screens and perform functional tests																
T22.4	Repeat Task 22.1 with mouse library and MEFs that express MycER.																
T22.5	The identified genes will be compared with the genes obtained in the screens in the other workpackages, in particular from WP																
T22.6	The expression of the identified genes in primary tumours will be analysed by database analysis and screening of multiple tumour arrays by ISH. Functional tests of positive inserts. Test of control shRNAs from Miz1S428A screen.																
T22.7	Gene copy numbers for selected overexpressed or non-expressed genes will be determined by FISH																
T 22.8	Perform candidate screen in neuroblastoma cells																
T22.9	Biochemical Characterization of AURKA effect in MYCN neuroblastoma cells																
T 22.10	Develop novel strategies for synthetic lethal screens in 96well format																
T 22.11	Screen shRNA kinase library in U2OS-MycEr cells																

	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 23	Tasks																
T23.1	Establishment of LTC-IC assays																
T23.2	Establishment of viral vectors that can be used to overexpress c-MYC																
T23.3	Production of high titre viruses to infect HSCs																
T23.4	Isolation of <i>PTEN</i> ^{ΔΔ} hematopoietic stem cells (HSCs)																
T23.5	Isolation of HSCs with the following genotypes: <i>p19ARF</i> ^{-/-} ; <i>PTEN</i> ^{ΔΔ} ; and <i>p19ARF</i> ^{-/-} ; <i>PTEN</i> ^{ΔΔ} doubles.																
T23.6	Generation of HSCs which overexpress c-Myc																
T23.7	Generation of HSCs which overexpress c-Myc on a <i>PTEN</i> ^{ΔΔ} background																
T23.8	Generation of HSCs which overexpress c-Myc on a <i>p19ARF</i> ^{-/-} background																
T23.9	Introduction of an inducible GFP allele into the different mutant backgrounds																
T 23.10	Determination of the time point by which each genotype has lost HSC activity in vitro																
T23.11	Determination of culture time c-Myc ⁺ Pten ^{ΔΔ} HSCs are lost																
T23.12	Carry out shRNA screen for vectors that promote c-Myc ⁺ Pten ^{ΔΔ} HSC self-renewal or survival																
T23.13	Functional tests of positive inserts																
T23.14	Sort different leukemic sub-populations and transplantation into NOD/SCID mice.																
T23.15	Microarray analysis of purified leukemic stem cells																
T23.16	Establishment of an inducible lentiviral system to overexpress or knock down genes in hematopoietic stem cells in vitro and in vivo																
T23.17	Establish the role of Pten in normal and malignant hematopoiesis																

	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 24	Tasks																

Third Annual Periodic Activity Report (2006)

[illegible][illegible][illegible]

Third Annual Periodic Activity Report (2006)

[illegible][illegible]

WP 28 (MCH) ended

WP 29 – see management report

[illegible]

	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 31	Tasks																
T 31.1	Build-up and maintain INTACT website																

	Months	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
WP 30	Tasks																
T 32.1	Installation of cellular inhibitory assay																

Third Annual Periodic Activity Report (2006)

[illegible]

Section 4 – Other issues

No issues to report on.

Section 5 – Plan for using and disseminating of knowledge (PUDK)

Partner 1, UKBH (WP 7, 15)

Section 1 – Exploitable knowledge at its use (month 36)

None of the results from the WPs have lead to exploitable results or patents

Section 2 -Dissemination of knowledge (month 36)

Publications (months 1-36)

2006

1. Bracken AP, Dietrich N, Pasini D, Hansen KH and **Helin K** (2006). Genome-wide mapping of Polycomb target genes unravels their role in cell-fate transitions. *Genes Dev* **20**, 1123-1136.
2. Cloos PAC, Christensen J, Agger K, Maiolica A, Rappsibler J, Antal T, Hansen KH and **Helin K** (2006) The putative oncogene GASC1/JMJD2c demethylates tri- and di-methylated lysine 9 on histone H3, *Nature* **442**, 307-311,
3. Fedele M, Visone R, De Martino I, Troncone G, Palmieri G, Ciarmiello A, Battista S, Pallante P, Arra C, Mellilo RM, **Helin K**, Croce CM and Fusco A. (2006) HMG2A induces pituitary tumorigenesis by enhancing E2F1 activity. *Cancer Cell*, **9**, 459-471.
4. Lu Z, Luo RZ, Peng H, Huang M, Nishimoto A, Hunt KK, **Helin K**, Liao WSL and Yu Y (2006). E2F-HDAC complexes negatively regulate the tumor suppressor gene ARHI in breast cancer. *Oncogene* **25**, 230-239.
5. Qiao H, Di Stefano L, Tian C, Li Y-Y, Qian X-P, Pang X-W, Li Y, McNutt MA, **Helin K**, Zhang Y, Chen W-F (2006) Human TFDP-3, a novel DP protein, inhibits DNA binding and transactivation by E2F. *J. Biol. Chem.* Oct 24; [Epub ahead of print].

2005

6. Adhikary S*, Marinoni F*, Hock A*, Hulleman E*, Popov N, Beier R, Bernard S, Quarto M, Capra M, Goettig S, Kogel U, Scheffner M, **Helin K**^, and Eilers M^ (2005). The E3 ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. *Cell* **123**, 409-421. (*Equal first authors, ^Corresponding authors)
7. Agger K, Santoni-Rugiu E, Holmberg C, Karlström O, and **Helin K** (2005). Conditional E2F1 activation causes testicular atrophy and intratubular neoplasia in transgenic mice. *Oncogene* **24**, 780-789.
8. Christensen J, Cloos P, Toftegaard U, Klinkenberg D, Bracken AP, Trinh E, Heeran M, Di Stefano L, and **Helin K** (2005). Characterization of E2F8, a novel E2F-like cell-cycle regulated repressor of E2F-activated transcription. *Nucleic Acids. Res.* **33**, 5458-5470.
9. Colombo E, Bonetti P, Denchi EL, Martinelli P, Zamponi R, Marine JM, **Helin K**, Falini B, and Pelicci PG (2005). Nucleophosmin is required for DNA integrity and p19Arf protein stability. *Mol. Cell. Biol.* **25**, 8874-8886.

Third Annual Periodic Activity Report (2006)

10. Denchi EL, Attwooll C, Pasini D and **Helin K** (2005). Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. *Mol. Cell. Biol.* **25**, 2660-2672.
11. Denchi EL and **Helin K** (2005). E2F1 is crucial for E2F-dependent apoptosis. *EMBO reports* **6**, 661-668.
12. Hulleman E and **Helin K** (2005). Molecular mechanisms in gliomagenesis. *Adv. Cancer Res.* **94**, 1-27.
13. Rohde M, Daugaard M, Jensen MH, **Helin K**, Nylandsted J and Jäättelä M (2005). Members of the heat shock protein 70 family promote cancer cell growth by different mechanisms. *Genes Dev* **19**, 570-582.

2004

14. Attwooll C, Denchi EL and **Helin K** (2004). The E2F family: specific functions and overlapping interests. *EMBO J* **23**, 4709-4716.
15. Attwooll C, Oddi S, Cartwright P, Prosperini E, Agger K, Steensgaard P, Wagener C, Sardet C, Moroni MC, and **Helin K** (2004) A novel repressive E2F6 complex containing the polycomb group proteins EPC1, that interacts with EZH2 in a proliferation-specific manner. *J. Biol. Chem.*, published Nov 8 as doi:10.1074/jbcM412509200. **280**, 1199-1208.
16. Ballabeni A, Melixitian M, Zamponi R, Masiero L, Marinoni F and **Helin K** (2004). Human Geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis. *EMBO J* **23**, 3122-3132.
17. Bracken AP, Ciro' M, Cocito A and **Helin K** (2004). E2F target genes: unraveling the biology. *Trends Biochem. Sci.* **29**, 409-417.
18. Danovi D, Meulmeester E, Pasini D, Migliorini D, Capra M, Frenk R, de Graaf P, Francoz S, Gasparini P, Gobbi A, **Helin K**, Pelicci PG, Jochemsen AG and Marine JG (2004). Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. *Mol. Cell. Biol.* **24**, 5835-5843.
19. Grassilli E, Ballabeni A, Maellaro E, Del Bello B, and **Helin K** (2004). Loss of Myc confers resistance to doxorubicin-induced apoptosis by preventing the activation of multiple serine protease- and caspase-mediated pathways. *J. Biol. Chem.* **279**, 21318-21326.
20. Jensen MR and **Helin K** (2004) OVCA1: emerging as a bona fide tumor suppressor. *Genes Dev* **18**, 245-248.
21. Melixetian M, Ballabeni A, Masiero L, Gasparini P, Zamponi R, Bartek J, Lukas J and **Helin K** (2004). Loss of Geminin induces rereplication in the presence of functional p53. *J. Cell Biol.* **165**, 473-482.
22. Melixetian M and **Helin K** (2004). Geminin: A major DNA replication safeguard in higher eukaryotes. *Cell Cycle* **3**, 1002-1004.
23. Pasini D, Bracken AP and **Helin K** (2004). Polycomb group proteins in cell cycle progression and cancer. *Cell Cycle* **3**, 396-400.
24. Pasini D, Bracken AP, Jensen MR, Denchi EL and **Helin K** (2004). The Polycomb Group protein Suz12 is essential for mouse development and for EZH2 Histone Methyltransferase activity. *EMBO J* **23**, 4061-4071.
25. Trinh E, Denchi EL and **Helin K** (2004). Naturally death-resistant precursor cells revealed as the origin of retinoblastoma. *Cancer Cell* **5**, 513-515.

Third Annual Periodic Activity Report (2006)

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
March 2006	Seminar, CR-UK London (UK)	Research	World wide	>10 ²	UKBH
March 2006	Seminar, ICL London (BE)	Research	World wide	>10 ²	UKBH
March 2006	Seminar, CSIC Madrid (ES)	Research	World wide	>10 ²	UKBH
March 2006	Lecture, UKBH Copenhagen (DK)	Research	World wide	20	UKBH
April 2006	Seminar, RUC Roskilde (DK)	Research	World wide	>10 ²	UKBH
April 2006	Seminar, Jakobiner-Club Copenhagen (DK)	Research	World wide	>10 ²	UKBH
May 2006	Seminar Program Cell Death Meeting, Copenhagen (DK)	Research	World wide	>10 ²	UKBH
June 2006	Seminar Workshop on Solid Tumors. La Grande Motte (FR)	Research	World wide	>10 ²	UKBH
June 2006	Lecture, UKBH Copenhagen (DK)	Research	World wide	25	UKBH
August 2006	Seminar, CSHL Cold Spring Harbor (US)	Research	World wide	>10 ²	UKBH
August 2006	Seminar, Harvard Medical School, Boston (US)	Research	World wide	>10 ²	UKBH
August 2006	Seminar, MRL Boston (US)	Research	Merck	50	UKBH
August 2006	Seminar, Flakkebjerg (DK)	Research	World wide	30	UKBH
September 2006	Seminar, University of Utrecht (NL)	Research	World wide	>10 ²	UKBH
September 2006	Seminar, Bioincubator meeting Lund (SE)	Research	World wide	>10 ²	UKBH
October 2006	Seminar, "Chromatin meeting", Marburg (DE)	Research	World wide	>10 ²	UKBH
October 2006	Seminar, University of Helsinki (FI)	Research	World wide	>10 ²	UKBH
November 2006	Seminar, "Meeting on Sentinel Node Biopsy", Rome (IT)	Research	World wide	>10 ²	UKBH
November 2006	Seminar, Symp. on Genomic and Epigenomic Alteration. Tokyo	Research	World wide	>10 ²	UKBH
December 2006	Seminar, EMBL Heidelberg (DE)	Research	World wide	>10 ²	UKBH
January	Seminar,	Research	World wide	>10 ²	UKBH

Third Annual Periodic Activity Report (2006)

2005	Copenhagen (DK)				
February 2005	Seminar, Gent (BE)	Research	World wide	>10 ²	UKBH
February 2005	Seminar Oxford (UK)	Research	World wide	>10 ²	UKBH
March 2005	Seminar, Workshop Berlin (DE)	Research	World wide	>10 ²	UKBH
March 2005	Seminar Copenhagen (DK)	Research	World wide	>10 ²	UKBH
March 2005	Seminar Conference, Copenhagen (DK)	Research General public	World wide	>10 ²	UKBH
April, 2005	Seminar Copenhagen (DK)	Research policy seminar	World wide	>10 ²	UKBH
April 2005	Seminar Paris (FR)	general public	World wide	>10 ²	UKBH
April 2005	Seminar Copenhagen (DK)	research	World wide	>10 ²	UKBH
May 2005	Seminar Copenhagen (DK)	Research policy seminar	World wide	>10 ²	UKBH
May 2005	Seminar Aarhus (DK)	Research	World wide	>10 ²	UKBH
May 2005	Seminar Copenhagen (DK)	Research	World wide	>10 ²	UKBH
June 2005	Seminar Stockholm (SE)	Research	World wide	>10 ²	UKBH
August 2005	Seminar Copenhagen (DK)	Research	World wide	>10 ²	UKBH
September 2005	Seminar Copenhagen (DK)	Research	World wide	>10 ²	UKBH
September 2005	Seminar Conference St. Odile (FR)	Research	World wide	>10 ²	UKBH
October 2005	Seminar Conference Copenhagen (DK)	Research policy seminar	World wide	>10 ²	UKBH
October 2005	Seminar Odense (DK)	Research	World wide	>10 ²	UKBH
December 2005	Seminar Conference Odense (DK)	Research	World wide	>10 ²	UKBH
December 2005	Seminar Malmö (SE)	Research	World wide	>10 ²	UKBH
January 2004	Seminar Danish Medical Society Copenhagen (DK)	Research Policy	World wide	>10 ²	IEO1
February 2004	Seminar, Karolinska Institutet, Stockholm (SE)	Research	World wide	>10 ²	IEO1

Third Annual Periodic Activity Report (2006)

April 2004	Seminar, ISREC Lausanne (CH)	Research	World wide	$>10^2$	IEO1
June 2004	Seminar, CRG Barcelona (ES)	Research	World wide	$>10^2$	IEO1
June 2004	Seminar, Conference Weizman, Israel	Research	World wide	$>10^2$	IEO1
July 2004	Seminar, Bioscience Conference, Glasgow (UK)	Research	World wide	$>10^3$	IEO1
September 2004	Seminar, Sympion Day, Copenhagen (DK)	Research	World wide	$>10^2$	IEO1
September 2004	Seminar, Biological Society, Copenhagen (DK)	Research	World wide	$>10^2$	IEO1
December 2004	Seminar, AACR conference Ft. Lauderdale (US)	Research	World wide	$>10^2$	IEO1
December 2004	Seminar, NYU New York (US)	Research	World wide	$>10^2$	IEO1

Section 3 – Publishable results

WP7 and WP15 have so far not given rise to exploitable results.

Partner 2, NKI1 (WP 5, 26)

Section 1 – Exploitable knowledge at its use

In the first year none of the research undertaken has led to exploitable results or patents.

Section 2 -Dissemination of knowledge (month 36)

Mention publications from the years 2004, 2005: *No publications have arisen from this work yet.*

Actual dates	type	Type of audience	Countries addressed	Size of audience	Partner involved
24/01/2006 Verona	seminar	Research	Italy	100	NKI1
06/04/2006 Turku	seminar	Research	Most EU	50	NKI1
12/04/2006 Boston	seminar	Research	US	100	NKI1
26/04/2006 Boston	seminar	oncology	US	100	NKI1
27/04/2006 Worchester	seminar	genetics	US	40	NKI1
02/05/2005 Dundee	seminar	oncology	EU	75	NKI1
11/05/2006 London	seminar	Oncology	EU	100	NKI1
18/05/2006 Bordeaux	conference	Oncology	EU	50	NKI1
30/05/2006 Boston	seminar	Research	USA	100	NKI1
02/06/2006 Copenhagen	conference	Research	EU	100	NKI1
08/06/2006 Rome	seminar	Research	Worldwide	50	NKI1
11/06/2006 Washington	conference	Research	Worldwide	300	NKI1
03/07/2006 Rome	PhD course	oncology	EU	30	NKI1
30/07/2006 Thailand	seminar	oncology	Asia	100	NKI1
10/09/2006 Gr. Rapids	conference	Research	worldwide	200	NKI1
29/10/2006 Singapore	seminar	Research	Asia	150	NKI1
15/11/2006 Berlin	conference	Research	Worldwide	150	NKI1
18/11/2006 Brunnen	conference	Research	Worldwide	75	NKI1
30/11/2006 London	conference	Oncology	GB	75	NKI1
13/12/2006 Munchen	seminar	Research	EU	150	NKI1
1/2005Cambridge GB	seminar	Oncology	GB	100	NKI1
2/2005 Banff Canada	conference	Oncology	Canada	300	NKI1
4/2005 AACR USA	congress	Oncology	USA	100	NKI1
5/2005 London	seminar	Oncology	GB	150	NKI1
6/2005 CSH	conference	Mol. Biol.	USA	400	NKI1
6/2005 Pezcoller	conference	Oncology	Italy	75	NKI1
9/2005 Strassbourg	Teaching	Geneticists	France	60	NKI1
10/2005 Heidelberg	conference	Dev. Biol.	Germany	250	NKI1
11/2005 Savannah	conference	Imaging	USA	25	NKI1
11/2005 Liverpool	Seminar	Oncology	GB	100	NKI1
11/2005 Budapest	conference	Oncology	Hungary	75	NKI1
1/2004, Kansas	seminar	medical	Most US	75	NKI1
2/2004,Basel	seminar	Mol. Biol.	Most EU	50	NKI1
2/2004,Keystone	conference	genetics	Most US	300	NKI1
3/2004,Milano	conference	medical	EU	150	Many
4/2004,USA	seminar	Genetics	US	75	NKI1
5/2004,France	conference	Cell Biol.	Most EU	25	NKI1
5/2004, UK	conference	Oncology	EU	200	NKI1
6/2004, Austin	conference	Oncology	Most US	300	NKI1
6/2004,Glasgow	conference	Mol. Biol.	Most EU	200	NKI1 NKI2
6/2004, Vienna	conference	oncology	EU	30	NKI1
6/2004, Berkeley	conference	oncology	EU	30	NKI1
9/2004, BarHarbor	conference	genetics	US, EU,	40	NKI1
9/2004,Malmo	seminar	oncology	Sweden	100	NKI1
9/2004,Paris	conference	oncology	EU	150	NKI1
10/2004,New York	conference	Oncology	USA	200	NKI1
10/2004,Amsterdam	conference	Oncology	EU, USA	25	NKI1

Third Annual Periodic Activity Report (2006)

12/2004,St.Louis	seminar	oncology	US	200	NKI1
------------------	---------	----------	----	-----	------

Section 3 – Publishable results

The integrated Project has so far not given rise to exploitable results.

Partner 2, NKI2 (WP 1, 2, 12, 25)

Section 1 – Exploitable knowledge at its use (month 36)

No exploitable results or patents

Section 2 -Dissemination of knowledge (month 36)

Publications (months 1-36)

2006

1. Brummelkamp, T.R., Fabius, A., Mullenders, J., Madiredjo, M., Velds, A., Kerkhoven, R.M., **Bernards, R.**, and Beijersbergen, R.L. (2006). An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. *Nature Chem. Biol.* **2**, 202-206.
2. **Bernards, R.**, Brummelkamp, T.R., and Beijersbergen, R.L. (2006). shRNA libraries and their use in cancer genetics. *Nature Methods* **3**, 701-706.

2005

3. Brummelkamp, T.R., Berns, K., Hijmans, E.M., Mullenders, J., Fabius, A., Heimerikx, M., Velds, A., Kerkhoven, R.M., Madiredjo, M., **Bernards, R.** and Beijersbergen, R.L. (2005). Functional identification of cancer-relevant genes through large-scale RNA interference screens in mammalian cells. In: *Cold Spring Harbor Symposia on Quantitative Biology Vol. 69: Epigenetics* p 439-445.

2004

4. Berns, K., Hijmans, E.M., Mullenders, J., Brummelkamp, T.R., Velds, A., Heimerikx, Kerkhoven, R. M., Madiredjo, M., Nijkamp, W., Weigelt, B., Agami, R., Ge, W., Cavet, G., Linsley, P.S., Beijersbergen, R.L. and **Bernards, R.** (2004). A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature*, 428, 431-437.

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
January, 2006	Conference, Florida, USA	Research	World wide	500	NKI2
January 2006	Conference, Vancouver, Canada	Research	World wide	300	NKI2
April 2006	Conference, Washington, USA	Research	World wide	700	NKI2
April 2006	Conference, Heidelberg, FRG	Research	World wide	250	NKI2
June 2006	Conference, New York, USA	Research	World wide	400	NKI2
October 2006	EU workshop Luxemburg	Research	EU mainly	100	NKI2
October 2006	Conference, Shanghai, PR China	Research	Asian	150	NKI2
November	Conference, Madrid, Spain	Research	EU	150	NKI2

Third Annual Periodic Activity Report (2006)

2006					
January 2005	Conference, Vienna (A)	Research	World wide	50	NKI2
January 2005	Conference, Santa Fe, USA	Research	World wide	350	NKI2
January 2005	Conference, Lausanne (Switzerland)	Research	World wide	200	NKI2
November, 2004	Conference, San Diego (USA)	Research	World wide	300	NKI2
March, 2005	Seminar, Boston (USA)	Research	World wide	300	NKI2
March, 2005	Conference, Keystone (USA)	Research	World wide	250	NKI2
April, 2005	Conference, Anaheim (USA)	Research	World wide	5000	NKI2
May, 2005	Conference, Turin (I)	Research	World wide	125	NKI2
June, 2005	Conference, Cold Spring Harbor, USA	Research	World wide	400	NKI2
June, 2005	Conference, Molde, Norway	Research	World wide	200	NKI2
July, 2005	Conference, Rhode Island, USA	Research	World wide	125	NKI2
August, 2005	Conference, Beijing, China	Research	World wide	300	NKI2
September, 2005	Seminar, Dresden, FRG	Research	World wide	200	NKI2
October, 2005	Conference, Houston, USA	Research	World wide	150	NKI2
January, 2004	Conference, Paris, France	medical	Most EU	150	NKI2
February, 2004	Conference, Paris, France	Oncology	Most EU	200	NKI2
February, 2004	Conference, Lausanne, France	oncology	Swiss, France	200	NKI2
March, 2004	Conference, USA	medical	USA	250	NKI2
March, 2004	Conference, USA	Mol. biol	USA, EU, Japan	350	NKI2
April, 2004	Conference, USA	Mol. Boil	USA, EU, Asia	2000	NKI2
April, 2004	Conference, USA	Mol. Biol	USA, EU, Asia	400	NKI2
May, 2004	Conference, USA	Mol. Biol	USA, EU	200	NKI2
June, 2004	Conference, USA	Mol. Biol.	Asia, USA, EU	400	NKI2
June, 2004	Conference, Glasgow, UK	oncology	EU, USA	200	NKI2
July, 2004	Conference, USA	oncology	EU USA Japan	150	NKI2
August, 2004	Conference, York, UK	genetics	UK	200	NKI2
September, 2004	Conference; Barcelona, Spain	oncology	Spain	100	NKI2
October, 2004	Conference, USA	Mol biol	USA, EU	200	NKI2
October, 2004	Conference, USA	oncology	USA	275	NKI2
October, 2004	Conference, Germany	Mol biol	EU, USA	125	NKI2
November, 2004	Conference, Viena, Austria	oncology	EU, USA, Asia	500	NKI2

Section 3 – Publishable results

WP 1

This workpackage has so far not given rise to exploitable results

All aims of this work package have been fulfilled. The technology is available and actually used by the partners of the consortium.

This workpackage is completed, no further tasks due.

WP 2

This workpackage has yielded a mouse shRNA library of some 30,000 vectors. Such a reagent will be a valuable tool for the entire European scientific community. Our technology transfer office in London is in talks with various distributors to make this reagent widely available at low cost to the academic investigators.

All aims of this workpackage have been fulfilled. The technology is available and actually used by the partners of the consortium.

This workpackage is completed, no further tasks due.

WP 12, 25

The integrated project has so far not given rise to exploitable results

Partner 2, NKI3 (WP3, 14)

Section 1 – Exploitable knowledge at its use (month 36)

In the second year none of the research undertaken has led to exploitable results or patents.

Section 2 -Dissemination of knowledge (month 36)

2006

1. Taghavi, P. and van Lohuizen, M. Developmental biology: two paths to silence merge. *Nature* 2006, 439, 794-795.
2. Tolhuis, B., de wit, E.*, Muijters, I.*, Teunissen, H., Talhout, W., van Steensel, B#. and van Lohuizen, M#. Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in *Drosophila*. *Nature Genet.* 2006; 38, 694-699. (*equal contribution; #corresponding authors).
3. Bruggeman, S.W.M and van Lohuizen, M. Controlling stem cell proliferation: CKIs at work. *Cell Cycle* 2006; 5, 1281-1285.
4. Gretel Buchwald, Petra van der Stoop, Oliver Weichenrieder, Anastassis Perrakis, Maarten van Lohuizen and Titia K. Sixma. Structure and E3-ligase activity of the Ring-Ring complex of polycomb proteins Bmi1 and Ring1b. *EMBO J.* 2006; 25, 2465-2474.
5. Fujimura YI, Isono KI, Vidal M, Endoh M, Kajita H, Mizutani-Koseki Y, Takihara Y, van Lohuizen M, Otte A, Jenuwein T, Deschamps J, Koseki H.
6. Distinct roles of Polycomb group gene products in transcriptionally repressed and active domains of Hoxb8. *Development.* 2006; 133, 2371-2381.
7. Pirrotta V, van Lohuizen M. Differentiation and gene regulation. Genomic programs and differentiation. *Curr Opin Genet Dev.* 2006;16, 443-446.
8. Oguro H, Iwama A, Morita Y, Kamijo T, van Lohuizen M, Nakauchi H. Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice. *J Exp Med.* 2006;203, 2247-2253.
9. Steele JC, Torr EE, Noakes KL, Kalk E, Moss PA, Reynolds GM, Hubscher SG, van Lohuizen M, Adams DH, Young LS. The polycomb group proteins, BMI-1 and EZH2, are tumour-associated antigens. *Br J Cancer.* 2006, 95, 1202-1211.
10. PMID: 17024127.
11. Sparmann A. and van Lohuizen M. Polycomb silencers control cell fate, development and cancer. *Nature Rev. Cancer* 2006, 6, 846-856.
12. Zencak D, Crippa SV, Tekaya M, Tanger E, Schorderet DE, Munier FL, van Lohuizen M, Arsenijevic Y. BMI1 loss delays photoreceptor degeneration in Rd1 mice. Bmi1 loss and neuroprotection in Rd1 mice. *Adv Exp Med Biol.* 2006;572:209-15
13. Hosokawa H, Kimura MY, Shinnakasu R, Suzuki A, Miki T, Koseki H, van Lohuizen M, Yamashita M, Nakayama T. Regulation of Th2 cell development by Polycomb group gene bmi-1 through the stabilization of GATA3. *J Immunol.* 2006 Dec 1;177(11):7656-64.

2005

14. Inmaculada Hernández-Muñoz*, Anders H. Lund*, Petra van der Stoop*, Erwin Boutsma, Inhua Muijters, Els Verhoeven, Dmitri A. Nusinow, Barbara Panning, York Marahrens and **Maarten van Lohuizen**. Stable X-chromosome inactivation involves dynamic recruitment of the PRC1 Polycomb complex, requires variant histone MACROH2A1 and the CULLIN3/SPOP Ubiquitin E3 Ligase. *Proc. Natl. Acad. Sci. USA* 2005, 102,7635-7640. (*These authors contributed equally)
15. Sophia W. M. Bruggeman*, Merel E. Valk-Lingbeek*, Petra P. M. van der Stoop, Jacqueline J. L. Jacobs, Karin Kieboom, Ellen Tanger, Danielle Hulsman, Carly Leung, Yvan Arsenijevic, Silvia Marino and **Maarten van Lohuizen**. Ink4a and Arf differentially affect cell proliferation and neural

Third Annual Periodic Activity Report (2006)

stem cell self-renewal in Bmi1 deficient mice. Genes Dev.2005, 19, 1438-1443. (*These authors contributed equally)

16. Dusan Zencak, Merel Lingbeek, Corinne Kostic, Meriem Tekaya, Ellen Tanger, Dana Hornfeld, Muriel Jaquet, Francis L. Munier, Daniel F. Schorderet, **Maarten van Lohuizen***, Yvan Arsenijevic*. Bmi1 loss produces an increase in astroglial cells and a decrease in neural stem cell population and proliferation. J. Neurosci. 2005, 25, 5774-5783. (*Corresponding authors)

2004

17. Leung, C*, Lingbeek, M*, Shakhova, O., Liu, J., Tanger, E., Saremaslani, P., **van Lohuizen, M#**, and Marino, S#. Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas. Nature 2004, 428, 337-341. (*These authors contributed equally; # co-corresponding authors).
18. Lund, A.H. and **van Lohuizen, M**. Polycomb complexes and silencing mechanisms. Curr. Opin. Cell Biol. 2004, 16, 239-264.
19. Carstens, M.J., Krempler, A., Triplett, A.A., **van Lohuizen, M**. and K.-U. Wagner. Cell cycle arrest and cell death are controlled by p53-dependent and p53-independent mechanisms in Tsg101-deficient cells. J. Biol Chem. 2004, 279, 35984-35994
20. Valk-Lingbeek, M.E., Bruggeman, S.W.M and **van Lohuizen, M**. Stem cells and cancer: the Polycomb connection. Cell 2004, 118, 409-418.
21. Lund, A.H. and **van Lohuizen, M**. Cancer epigenetics. Genes Dev. 2004, 18, 2315-2335.
22. Inhua Muyrers-Chen, Inmaculada Hernández-Muñoz, Anders H. Lund, Merel E. Valk-Lingbeek, Petra van der Stoop, Erwin Boutsma, Bas Tolhuis, Sophia W.M. Bruggeman, Panthea Taghavi, Els Verhoeven, Danielle Hulsman, Sonja Noback, Ellen Tanger, Hans Theunissen and **Maarten van Lohuizen**. Emerging roles of Polycomb silencing in X-inactivation and stem cell maintenance. Cold Spring Harb. Symp. Quant. Biol. 69, in press
23. Iwama, A., Oguro, H., Negishi, M., Kato, Y., Morita, Y., Tsukui, H., Ema, H., Kamijo, T., Katoh-Fukui, Y., Koseki, H., **van Lohuizen, M**. and Nakauchi, H. Enhanced Self-renewal of hematopoietic stem cells mediated by the Polycomb gene product Bmi-1. Immunity 2004, 21, 843-851.

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
January 2006	Seminar, Keystone, USA	Research	World wide	>10 ²	NKI3
February 2006	AACR stem cell Workshop, USA	Research, Policy seminar	World wide	>10 ²	NKI3
February 2005	Seminar Odense (DK)	Research	World wide	>10 ²	NKI3
March 2006	Seminar, Heidelberg (GM)	Research	World wide	>10 ²	NKI3
March 2006	Seminar, Lilly foundation CNIO (SP)	Research	World wide	>10 ²	NKI3
March 2006	Seminar, BSCB/BSDB meeting, (UK)	Research	World wide	>10 ²	NKI3
April, 2006	Seminar, 97 th AACR Meeting, (USA)	Research	World wide	>10 ³	NKI3
May 2006	Seminar Paterson, Manchester, (UK)	Research	World wide	>10 ²	NKI3

Third Annual Periodic Activity Report (2006)

June 2006	Seminar CRG Barcelona, (SP)	Research	World wide	$>10^2$	NKI3
June 2006	Seminar 2nd NoE Epigenome conference, Napoli, (IT)	Research	World wide	$>10^2$	NKI3
August 2006	Seminar GRC, Smithfield, (USA)	Research	World wide	$>10^2$	NKI3
August 2006	Seminar EMBO/ SNF, Arolla, (SW)	Research	World wide	$>10^2$	NKI3
August 2006	ESTOOLS FP6 IP, Sheffield, UK	Research	World wide	$>10^2$	NKI3
August 2006	Seminar BSS stem cell symposium, Kiel (GM)	Research	World wide	$>10^2$	NKI3
September 2006	EMBO symposium, Catanzaro, (IT)	Research	World wide	$>10^2$	NKI3
October 2006	Seminar NCRI Conference, Birmingham, UK	Research	World wide	$>10^2$	NKI3
October 2006	Seminar, 6 th ISREC/EPFL conference, Lausanne, (SW)	Research	World wide	$>10^2$	NKI3
October 2006	Seminar EMBL, Heidelberg, (GM)	Research	World wide	$>10^2$	NKI3
November 2006	Schering stem cell seminar, Berlin, (GM)	Research	World wide	$>10^2$	NKI3
December 2006	Stem cell Seminar, WTCSCR/Nature Cambridge, (UK)	Research	World wide	$>10^2$	NKI3
March, 2005	13th International AEK/AIO Congress German Cancer Society, Wurzburg (Germany)	Research	World wide	>102	NKI3
April, 2005	96th AACR Annual Meeting, Symposium speaker, Anaheim (USA)	Research	World wide	>102	NKI3
May, 2005	6th Nature Horizon Symposium on Epigenetics, Black Point Inn, Maine (USA)	Research	World wide	>102	NKI3
June, 2005	70th Cold Spring Harbor Symposium on Molecular approaches to controlling cancer, CSHL, New York (USA)	Research	World wide	>102	NKI3
June, 2005	Seminar, Gurdon Institute, Cambridge (UK)	Research	World wide	>102	NKI3
June, 2005	Seminar Marie Curie Institute Pasteur, Paris (France)	Research	World wide	>102	NKI3

Third Annual Periodic Activity Report (2006)

July, 2005	FASEB meeting on Chromatin & Transcription, Snow Mass, Colorado (USA)	Research	World wide	>102	NKI3
July, 2005	Gordon Conference on Cancer Models & Mechanisms, Smithfield (USA)	Research	World wide	>102	NKI3

Section 3 – Publishable results

The integrated Project has so far not given rise to exploitable results.

Partner 3, CNIO1 (WP 8, 9, 16)

Section 1 – Exploitable knowledge at its use (month 36)

None of the results from the WPs have lead to exploitable results or patents

Section 2 -Dissemination of knowledge (month 36)

Publications (months 1-36)

2006

1. Benetti R, Garcia-Cao M, **Blasco MA**. Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. *Nat Genet.* 2007 Jan 21; [Epub ahead of print]
2. Blanco R, Munoz P, Flores JM, Klatt P, **Blasco MA**. Telomerase abrogation dramatically accelerates TRF2-induced epithelial carcinogenesis. *Genes Dev.* 2007 Jan 15;21(2):206-20
3. Rebuzzini P, Martinelli P, **Blasco MA**, Giulotto E, Mondello C. Inhibition of gene amplification in telomerase deficient immortalized mouse embryonic fibroblasts. *Carcinogenesis.* 2006 Sep 14; [Epub ahead of print]
4. Flores I, Evan G, **Blasco MA**. Genetic analysis of myc and telomerase interactions in vivo. *Mol Cell Biol.* 2006 Aug;26(16):6130-8
5. Perez-Rivero G, Ruiz-Torres MP, Rivas-Elena JV, Jerkic M, Diez-Marques ML, Lopez-Novoa JM, **Blasco MA**, Rodriguez-Puyol D. Mice deficient in telomerase activity develop hypertension because of an excess of endothelin production. *Circulation.* 2006 Jul 25;114(4):309-17
6. Flores I, Benetti R, **Blasco MA**. Telomerase regulation and stem cell behaviour. *Curr Opin Cell Biol.* 2006 Jun;18(3):254-60
7. Garcia-Cao I, Garcia-Cao M, Tomas-Loba A, Martin-Caballero J, Flores JM, Klatt P, **Blasco MA**, Serrano M. Increased p53 activity does not accelerate telomere-driven ageing. *EMBO Rep.* 2006 May;7(5):546-52
8. Munoz P, Blanco R, **Blasco MA**. Role of the TRF2 telomeric protein in cancer and ageing. *Cell Cycle.* 2006 Apr;5(7):718-21
9. Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M, **Blasco MA**. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. *Nat Cell Biol.* 2006 Apr;8(4):416-24
10. Geserick C, **Blasco MA**. Novel roles for telomerase in aging. *Mech Ageing Dev.* 2006 Jun;127(6):579-83
11. Geserick C, Tejera A, Gonzalez-Suarez E, Klatt P, **Blasco MA**. Expression of mTert in primary murine cells links the growth-promoting effects of telomerase to transforming growth factor-beta signaling. *Oncogene.* 2006 Jul 20;25(31):4310-9

2005

12. Martin M, Genesca A, Latre L, Jaco I, Taccioli GE, Egozcue J, **Blasco MA**, Iliakis G, Tusell L. Postreplicative joining of DNA double-strand breaks causes genomic instability in DNA-PKcs-deficient mouse embryonic fibroblasts. *Cancer Res.* 2005 Nov 15;65(22):10223-32
13. Rodriguez S, Goyanes V, Segrelles E, **Blasco MA**, Gosalvez J, Fernandez JL. Critically short telomeres are associated with sperm DNA fragmentation. *Fertil Steril.* 2005 Oct;84(4):843-5
14. Munoz P, Blanco R, Flores JM, **Blasco MA**. XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer. *Nat Genet.* 2005 Oct;37(10):1063-71
15. **Blasco MA**. Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet.* 2005 Aug;6(8):611-22
16. Flores I, Cayuela ML, **Blasco MA**. Effects of telomerase and telomere length on epidermal stem cell behavior. *Science.* 2005 Aug 19;309(5738):1253-6
17. Gonzalo S, **Blasco MA**. Role of Rb family in the epigenetic definition of chromatin. *Cell Cycle.* 2005 Jun;4(6):752-5
18. Franco S, Canela A, Klatt P, **Blasco MA**. Effectors of mammalian telomere dysfunction: a comparative transcriptome analysis using mouse models. *Carcinogenesis.* 2005 Sep;26(9):1613-26
19. Keefe DL, Franco S, Liu L, Trimarchi J, Cao B, Weitzen S, Agarwal S, **Blasco MA**. Telomere length predicts embryo fragmentation after in vitro fertilization in women--toward a telomere theory of reproductive aging in women. *Am J Obstet Gynecol.* 2005 Apr;192(4):1256-60
20. **Blasco MA**. Mice with bad ends: mouse models for the study of telomeres and telomerase in cancer and aging. *EMBO J.* 2005 Mar 23;24(6):1095-103
21. Gonzalo S, Garcia-Cao M, Fraga MF, Schotta G, Peters AH, Cotter SE, Eguia R, Dean DC, Esteller M, Jenuwein T, **Blasco MA**. Role of the RB1 family in stabilizing histone methylation at constitutive heterochromatin. *Nat Cell Biol.* 2005 Apr;7(4):420-8
22. Cayuela ML, Flores JM, **Blasco MA**. The telomerase RNA component Terc is required for the tumour-promoting effects of Tert overexpression. *EMBO Rep.* 2005 Mar;6(3):268-74
23. Gonzalez-Suarez E, Geserick C, Flores JM, **Blasco MA**. Antagonistic effects of telomerase on cancer and aging in K5-mTert transgenic mice. *Oncogene.* 2005 Mar 24;24(13):2256-70
24. Dixon IM, Lopez F, Esteve JP, Tejera AM, **Blasco MA**, Pratviel G, Meunier B. Porphyrin derivatives for telomere binding and telomerase inhibition. *Chembiochem.* 2005 Jan;6(1):123-32

2004

25. Espejel S, Klatt P, Menissier-de Murcia J, Martin-Caballero J, Flores JM, Taccioli G, de Murcia G, **Blasco MA**. Impact of telomerase ablation on organismal viability, aging, and tumorigenesis in mice lacking the DNA repair proteins PARP-1, Ku86, or DNA-PKcs. *J Cell Biol*. 2004 Nov 22;167(4):627-38.
26. Jaco I, Munoz P, **Blasco MA**. Role of human Ku86 in telomere length maintenance and telomere capping. *Cancer Res*. 2004 Oct 15;64(20):7271-8.
27. Latre L, Genesca A, Martin M, Ribas M, Egozcue J, **Blasco MA**, Tusell L. Repair of DNA broken ends is similar in embryonic fibroblasts with and without telomerase. *Radiat Res*. 2004 Aug;162(2):136-42.
28. Franco S, van de Vrugt HJ, Fernandez P, Aracil M, Arwert F, **Blasco MA**. Telomere dynamics in Fancg-deficient mouse and human cells. *Blood*. 2004 Dec 15;104(13):3927-35.
29. Ferron S, Mira H, Franco S, Cano-Jaimez M, Bellmunt E, Ramirez C, Farinas I, **Blasco MA**. Telomere shortening and chromosomal instability abrogates proliferation of adult but not embryonic neural stem cells. *Development*. 2004 Aug;131(16):4059-70.
30. **Blasco MA**. Carcinogenesis Young Investigator Award. Telomere epigenetics: a higher-order control of telomere length in mammalian cells. *Carcinogenesis*. 2004 Jul;25(7):1083-7.
31. Canela A, Martin-Caballero J, Flores JM, **Blasco MA**. Constitutive expression of tert in thymocytes leads to increased incidence and dissemination of T-cell lymphoma in Lck-Tert mice. *Mol Cell Biol*. 2004 May;24(10):4275-93.
32. Tarsounas M, Munoz P, Claas A, Smiraldi PG, Pittman DL, **Blasco MA**, West SC. Telomere maintenance requires the RAD51D recombination/repair protein. *Cell*. 2004 Apr 30;117(3):337-47.
33. Espejel S, Martin M, Klatt P, Martin-Caballero J, Flores JM, **Blasco MA**. Shorter telomeres, accelerated ageing and increased lymphoma in DNA-PKcs-deficient mice. *EMBO Rep*. 2004 May;5(5):503-9.
34. Liu L, Franco S, Spyropoulos B, Moens PB, **Blasco MA**, Keefe DL. Irregular telomeres impair meiotic synapsis and recombination in mice. *Proc Natl Acad Sci U S A*. 2004 Apr 27;101(17):6496-501.
35. Villa A, Navarro-Galve B, Bueno C, Franco S, **Blasco MA**, Martinez-Serrano A. Long-term molecular and cellular stability of human neural stem cell lines. *Exp Cell Res*. 2004 Apr 1;294(2):559-70.
36. Liu L, DiGirolamo CM, Navarro PA, **Blasco MA**, Keefe DL. Telomerase deficiency impairs differentiation of mesenchymal stem cells. *Exp Cell Res*. 2004 Mar 10;294(1):1-8.
37. Garcia-Cao M, O'Sullivan R, Peters AH, Jenuwein T, **Blasco MA**. Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. *Nat Genet*. 2004 Jan;36(1):94-9.
38. Poch E, Carbonell P, Franco S, Diez-Juan A, **Blasco MA**, Andres V. Short telomeres protect from diet-induced atherosclerosis in apolipoprotein E-null mice. *FASEB J*. 2004 Feb;18(2):418-20.

Third Annual Periodic Activity Report (2006)

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
November, 2006	Conference Josef Steiner Cancer Conference (Madrid, Spain)	Research	World wide	$>10^2$	CNIO1
July, 2006	Seminar CEA (Paris, France)	Research	Europe	50	CNIO1
July, 2006	Seminar Curie Institute (Paris, France)	Research	Europe	50	CNIO1
May, 2006	Press "Diario médico"	General public	Spain	$>10^3$	CNIO1
May, 2006	Press "Gaceta médica"	General public	Spain	$>10^3$	CNIO1
May, 2006	Press release Agencia "Europa press"	General public	Spain South America	$>10^6$	CNIO1
May, 2006	Press "Expansión"	General public	Spain	$>10^4$	CNIO1
May, 2006	Press "Perspectives" in The Lancet	Research	World wide	$>10^4$	CNIO1
May, 2006	Seminar 2nd IFOM-IEO Campus Meeting on Cancer (Milan, Italy)	Research	Europe	50	CNIO1 IEO1
May, 2006	Press "Research highlight in brief" in Nature Reviews Cancer	Research	World wide	$>10^5$	CNIO1
May, 2006	Internet Madrimsd.org	General public	Spain	$>10^3$	CNIO1
May, 2006	Press release Agencia "Salute Europa"	General public	Italy	$>10^4$	CNIO1
May, 2006	Internet Yahoo Italia, canal "Salute"	General public	Italy	$>10^5$	CNIO1
May, 2006	Press "El País"	General public	Spain	$>10^5$	CNIO1
April, 2006	Internet Azprensa.com	Biotechnology	Spain South America	$>10^3$	CNIO1
April, 2006	Conference AACR 97 th Annual Meeting (Washington DC, USA)	Research	World wide	$>10^2$	CNIO1
March, 2006	Internet Madrimsd.org	General public	Spain	$>10^3$	CNIO1
March, 2006	Internet tecnociencia.es	Biotechnology General public	Spain	$>10^3$	CNIO1
March,	Press	General	Spain	$>10^3$	CNIO1

Third Annual Periodic Activity Report (2006)

2006	“Diario médico”	public			
March, 2006	Press “Latest highlights” in Nature Cell Biology	Research	World wide	$>10^5$	CNIO1
March, 2006	Press “Bioforum Europe”	Research Biotechnology	Europe	$>10^4$	CNIO1
January, 2006	Internet terra.es	General public	Spain South America	$>10^6$	CNIO1
January, 2006	Conference Second Annual INTACT meeting (Madrid, Spain)	Research	World wide	50	all partners
November, 2005	Press “Diario Médico”	General public	Spain	$>10^5$	CNIO1
November, 2005	Conference, CNIO Cancer Conference “Cancer and Aging” (Madrid, Spain)	Research	World wide	$>10^2$	CNIO1
November, 2005	Workshop, “Current trends in medicine” (Baeza, Spain)	Research	World wide	$>10^2$	CNIO1
October, 2005	Seminar The Netherlands postdoc retreat “Making the right moves” (Egmond an Zee, Netherlands)	Research	Europe	$>10^2$	CNIO1 Pasteur
October, 2005	Seminar Center of Dev. Biol. (Utrecht, Netherlands)	Research	Europe	50	CNIO1 Pasteur
September, 2005	Conference II SENS meeting, (Cambridge, UK)	Research	Europe	$>10^2$	CNIO1
August, 2005	Press “Research Highlights in brief” in Nature Medicine	Research	World wide	$>10^6$	CNIO1
August, 2005	Press “Highlights in brief” in Nature Reviews Cancer	Research	World wide	$>10^6$	CNIO1
August, 2005	Press “Diario ABC”	General public	Spain	$>10^5$	CNIO1
August, 2005	Press release “El Mundo”	General public	Spain	$>10^6$	CNIO1
August, 2005	Press “La Vanguardia”	General public	Spain	$>10^5$	CNIO1
August, 2005	Press “Diario Médico”	General public	Spain	$>10^5$	CNIO1
June, 2005	Conference FASEB Conference	Research	USA	$>10^2$	CNIO1 Pasteur

Third Annual Periodic Activity Report (2006)

	"Chromatin"(CA, USA)				
May, 2005	Seminar Pasteur Institute (Paris, FR)	Research	Europe	50	CNIO1 Pasteur
March, 2005	Press "Diario de Mallorca"	General public	Spain	$>10^5$	CNIO1
March, 2005	Press "Diario Médico"	General public	Spain	$>10^5$	CNIO1
January, 2005	Conference 1st INTACT meeting, (Vienna, Austria)	Research	Europe	50	all partners
December, 2004	Conference Juan March Workshop (Madrid, Spain) "Recombinational DNA Repair and its Links with DNA Replication and Chromosome Maintenance"	Research	World wide	$>10^2$	CNIO1
December, 2004	Conference Stanford Medical College (CA, USA)	Research	USA	$>10^2$	CNIO1
December, 2004	Press "El País"	General public	Spain	$>10^6$	CNIO1
November, 2004	Press "EuroBiotech News", N°11, Vol.3	Industry (Biotech sector)	Europe	$>10^6$	CNIO1
November, 2004	Press "El País"	General public	Spain	$>10^6$	CNIO1
November, 2004	Conference AACR conference (San Francisco, USA) "The Role of Telomeres and Telomerase in Cancer"	Research	World wide	$>10^3$	CNIO1
October, 2004	Press "Science next wave"	Research	World wide	$>10^5$	CNIO1
October, 2004	Press "Naturejobs" Vol. 431, p. 1019	Research	World wide	$>10^5$	CNIO1
October, 2004	Press "Diario Siglo XXI"	General public	Spain	$>10^5$	CNIO1
October, 2004	Press "Diario ABC"	General public	Spain	$>10^5$	CNIO1
October, 2004	Press "El Mundo"	General public	Spain	$>10^6$	CNIO1
October, 2004	Press "La Razón"	General public	Spain	$>10^5$	CNIO1
October, 2004	Press "Science", Vol.306, p.224	Research	World wide	$>10^5$	CNIO1
October,	Press	General	Spain	$>10^6$	CNIO1

Third Annual Periodic Activity Report (2006)

2004	"El País"	public			
September, 2004	Press release "EMBO"	Research & general public	World wide	$>10^6$	CNIO1
May, 2004	Press "La Razón"	General public	Spain	$>10^5$	CNIO1
May, 2004	Press "ScienceNOW"	Research	World wide	$>10^5$	CNIO1
May, 2004	Press "Diario Médico"	General public	Spain	$>10^5$	CNIO1
February, 2004	Conference Juan March Workshop (Madrid, Spain) "Molecular Cross Talk Among Chromosome Fragility Syndromes"	Research	World wide	$>10^2$	CNIO1

Section 3 – Publishable results

WP8, WP9, and WP16 have so far not given rise to exploitable results.

Partner 3, CNIO2 (WP 20)

Section 1 – Exploitable knowledge at its use (month 36)

None of the results from the WPs have lead to exploitable results or patents

Section 2 -Dissemination of knowledge (month 36)

Publications (months 1-36)

2006

1. Efeyan A, Garcia-Cao I, Herranz D, Velasco-Miguel S, **Serrano M**. Tumour biology: Policing of oncogene activity by p53. *Nature*. 2006 Sep 14;443(7108):159
2. Efeyan A, Collado M, Velasco-Miguel S, **Serrano M**. Genetic dissection of the role of p21(Cip1/Waf1) in p53-mediated tumour suppression. *Oncogene*. 2006 Sep 11; [Epub ahead of print]
3. Nieto M, Barradas M, Criado LM, Flores JM, **Serrano M**, Llano E. Normal cellular senescence and cancer susceptibility in mice genetically deficient in Ras-induced senescence-1 (Ris1). *Oncogene*. 2006 Sep 11; [Epub ahead of print]
4. Garcia MA, Collado M, Munoz-Fontela C, Matheu A, Marcos-Villar L, Arroyo J, Esteban M, **Serrano M**, Rivas C. Antiviral action of the tumor suppressor ARF. *EMBO J*. 2006 Sep 20;25(18):4284-92
5. Llanos S, Efeyan A, Monsech J, Dominguez O, **Serrano M**. A high-throughput loss-of-function screening identifies novel p53 regulators. *Cell Cycle*. 2006 Aug;5(16):1880-5.
6. Zeini M, Traves PG, Lopez-Fontal R, Pantoja C, Matheu A, **Serrano M**, Bosca L, Hortelano S. Specific contribution of p19(ARF) to nitric oxide-dependent apoptosis. *J Immunol*. 2006 Sep 1;177(5):3327-36
7. Gonzalez S, **Serrano M**. A new mechanism of inactivation of the INK4/ARF locus. *Cell Cycle*. 2006 Jul;5(13):1382-4
8. Collado M, **Serrano M**. The power and the promise of oncogene-induced senescence markers. *Nat Rev Cancer*. 2006 Jun;6(6):472-6
9. Gonzalez L, Freije JM, Cal S, Lopez-Otin C, **Serrano M**, Palmero I. A functional link between the tumour suppressors ARF and p33ING1. *Oncogene*. 2006 Aug 24;25(37):5173-9
10. Garcia-Cao I, Garcia-Cao M, Tomas-Loba A, Martin-Caballero J, Flores JM, Klatt P, Blasco MA, **Serrano M**. Increased p53 activity does not accelerate telomere-driven ageing. *EMBO Rep*. 2006 May;7(5):546-52
11. Gonzalez S, Klatt P, Delgado S, Conde E, Lopez-Rios F, Sanchez-Cespedes M, Mendez J, Antequera F, **Serrano M**. Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. *Nature*. 2006 Mar 30;440(7084):702-6
12. De la Cueva E, Garcia-Cao I, Herranz M, Lopez P, Garcia-Palencia P, Flores JM, **Serrano M**, Fernandez-Piqueras J, Martin-Caballero J. Tumorigenic activity of p21Waf1/Cip1 in thymic lymphoma. *Oncogene*. 2006 Jul 6;25(29):4128-32
13. Silva J, Silva JM, Barradas M, Garcia JM, Dominguez G, Garcia V, Pena C, Gallego I, Espinosa R, **Serrano M**, Bonilla F. Analysis of the candidate tumor suppressor Ris-1 in primary human breast carcinomas. *Mutat Res*. 2006 Feb 22;594(1-2):78-85

2005

14. Collado M, **Serrano M**. The senescent side of tumor suppression. *Cell Cycle*. 2005 Dec;4(12):1722-4
15. Matheu A, Klatt P, **Serrano M**. Regulation of the INK4a/ARF locus by histone deacetylase inhibitors. *J Biol Chem*. 2005 Dec 23;280(51):42433-41
16. Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, Benguria A, Zaballos A, Flores JM, Barbacid M, Beach D, **Serrano M**. Tumour biology: senescence in premalignant tumours. *Nature*. 2005 Aug 4;436(7051):642
17. Garcia-Cao I, Duran A, Collado M, Carrascosa MJ, Martin-Caballero J, Flores JM, Diaz-Meco MT, Moscat J, **Serrano M**. Tumour-suppression activity of the proapoptotic regulator Par4. *EMBO Rep*. 2005 Jun;6(6):577-83
18. Munoz-Fontela C, Angel Garcia M, Garcia-Cao I, Collado M, Arroyo J, Esteban M, **Serrano M**, Rivas C. Resistance to viral infection of super p53 mice. *Oncogene*. 2005 Feb 14; [Epub ahead of print]
19. Gonzalez S, Perez-Perez MM, Hernando E, **Serrano M**, Cordon-Cardo C. p73beta-Mediated apoptosis requires p57kip2 induction and IEX-1 inhibition. *Cancer Res*. 2005 Mar 15;65(6):2186-92
20. Pantoja C, de Los Rios L, Matheu A, Antequera F, **Serrano M**. Inactivation of imprinted genes induced by cellular stress and tumorigenesis. *Cancer Res*. 2005 Jan 1;65(1):26-33
21. Goeman F, Thormeyer D, Abad M, **Serrano M**, Schmidt O, Palmero I, Baniahmad A. Growth inhibition by the tumor suppressor p33ING1 in immortalized and primary cells: involvement of two silencing domains and effect of Ras. *Mol Cell Biol*. 2005 Jan;25(1):422-31
22. Matheu A, Pantoja C, Efeyan A, Criado LM, Martin-Caballero J, Flores JM, Klatt P, **Serrano M**. Increased gene dosage of Ink4a/Arf results in cancer resistance and normal aging. *Genes Dev*. 2004 Nov 15;18(22):2736-46

2004

23. Matheu A, Pantoja C, Efeyan A, Criado LM, Martin-Caballero J, Flores JM, Klatt P, **Serrano M**. Increased gene dosage of Ink4a/Arf results in cancer resistance and normal aging. *Genes Dev*. 2004 Nov 15;18(22):2736-46
24. Martin-Caballero J, Flores JM, Garcia-Palencia P, Collado M, **Serrano M**. Different cooperating effect of p21 or p27 deficiency in combination with INK4a/ARF deletion in mice. *Oncogene*. 2004 Oct 28;23(50):8231-7
25. Duran A, **Serrano M**, Leitges M, Flores JM, Picard S, Brown JP, Moscat J, Diaz-Meco MT. The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. *Dev Cell*. 2004 Feb;6(2):303-9
26. Nieto M, Samper E, Fraga MF, Gonzalez de Buitrago G, Esteller M, **Serrano M**. The absence of p53 is critical for the induction of apoptosis by 5-aza-2'-deoxycytidine. *Oncogene*. 2004 Jan 22;23(3):735-43

Third Annual Periodic Activity Report (2006)

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
October, 2006	Seminar, Gulbenkian Science Institute (Lisbon, Portugal)	Research	Europe	50	CNIO2
October, 2006	Seminar, Dana-Farber Cancer Institute (Boston USA)	Research	USA	50	CNIO2
October, 2006	Press “Research Highlights” in Nature Reviews Cancer 2006, Volume 6, p.745	research	World wide	$>10^5$	CNIO2
October, 2006	Press “El País”	general	Spain	$>10^5$	CNIO2
September, 2006	Internet “Gaceta Médica Digital”	Medical doctors	Spain	$>10^3$	CNIO2
September, 2006	Internet “lne.es”	general	Spain	$>10^3$	CNIO2
September, 2006	Internet “terra.es”	general	Spain South America	$>10^3$	CNIO2
September, 2006	Internet “elcorreodigital.com”	general	Spain	$>10^3$	CNIO2
September, 2006	Press release “Europa press”	general	World wide	$>10^6$	CNIO2
September, 2006	Press release “Agencia efe”	general	Spain South America	$>10^6$	CNIO2
September, 2006	Press “El Correo Gallego”	general	Spain	$>10^4$	CNIO2
September, 2006	Press “Diario de León”	general	Spain	$>10^4$	CNIO2
September, 2006	Press “Diario ABC”	general	Spain	$>10^5$	CNIO2
September, 2006	Press “Faro de Vigo”	general	Spain	$>10^4$	CNIO2
September, 2006	Press “Diario Sur”	general	Spain	$>10^4$	CNIO2
September, 2006	Press “El Comercio”	general	Spain	$>10^4$	CNIO2
September, 2006	Press “La Gaceta”	general	Spain	$>10^4$	CNIO2
September, 2006	Press “El País”	general	Spain	$>10^5$	CNIO2
September, 2006	Press “La Verdad”	general	Spain	$>10^4$	CNIO2

Third Annual Periodic Activity Report (2006)

September, 2006	Internet “El Correo Digital”	general	Spain	$>10^4$	CNIO2
September, 2006	Press “Diario médico”	medical doctors	Spain	$>10^3$	CNIO2
September, 2006	Press “News & Views” in Nature 2006, Volume 443, p.153-154	research	World wide	$>10^5$	CNIO2
September, 2006	Press “Editor’s choice” in Sci. STKE, 2006, issue 353, p. tw318	research	World wide	$>10^5$	CNIO2
July, 2006	Seminar, Pasteur Institute (Paris, France)	research	Europe	50	CNIO2
July, 2006	Seminar, Curie Institute (Paris, France)	research	Europe	50	CNIO2
June, 2006	Press Editorial in Mol Cancer Ther 2006;5(6). June 2006	research	World wide	$>10^3$	CNIO2
June, 2006	Press “Research Highlights” in Nat. Rev. Cancer 2006, Volume 6, p.418	research	World wide	$>10^5$	CNIO2
March, 2006	Internet “Doymo Farma”	pharmacists	South America	$>10^3$	CNIO2
March, 2006	Internet “Los Tiempos.com”	general	South America	$>10^6$	CNIO2
April, 2006	Internet “Faculty of 1000 Biology”	research	World wide	$>10^5$	CNIO2
January, 2006	Conference Second Annual INTACT meeting (Madrid, Spain)	research	World wide	50	all partners
November, 2005	Conference, CNIO Cancer Conference “Cancer and Aging” (Madrid, Spain)	research	World wide	$>10^2$	CNIO2
August, 2005	Press “La Opinión”	general	Spain	$>10^4$	CNIO2
August, 2005	Press “Diario de Ibiza”	general	Spain	$>10^4$	CNIO2
August, 2005	Press “Diario de Navarra”	general	Spain	$>10^4$	CNIO2
August, 2005	Press “La Crónica de Hoy”	general	Mexico	$>10^4$	CNIO2
August, 2005	Press “Correo del Caroní”	general	Venezuela	$>10^4$	CNIO2

Third Annual Periodic Activity Report (2006)

August, 2005	Press “Segre”	general	Spain	$>10^4$	CNIO2
August, 2005	Press “El Diario Montañes”	general	Spain	$>10^4$	CNIO2
August, 2005	Press “El Correo”	general	Spain	$>10^4$	CNIO2
August, 2005	Internet “consumer.es”	general	Spain	$>10^5$	CNIO2
August, 2005	Press “La Vanguardia”	general	Spain	$>10^5$	CNIO2
August, 2005	Press “Faro de Vigo”	general	Spain	$>10^3$	CNIO2
August, 2005	Internet “Estrella Digital”	general	Spain	$>10^5$	CNIO2
August, 2005	Press “El Periódico”	general	Spain	$>10^4$	CNIO2
August, 2005	Press “Diario Córdoba”	general	Spain	$>10^4$	CNIO2
August, 2005	Press “Diario Sur”	general	Spain	$>10^4$	CNIO2
August, 2005	Internet “larioja.com”	general	Spain	$>10^4$	CNIO2
August, 2005	Internet “terra.es”	general	Spain	$>10^6$	CNIO2
August, 2005	Press “Expansión”	general	Spain	$>10^4$	CNIO2
June, 2005	Conference, Cold Spring Harbor Laboratory’s 70 th Symposium (Cold Spring Harbor, USA)	Research	Europe	$>10^2$	CNIO2
April, 2005	Conference, AACR’s 96 th Annual Meeting (Anaheim, USA)	Research	USA	$>10^2$	CNIO2
January, 2005	Conference, French Society on Cancer (Paris, France)	Research	Europe	$>10^2$	CNIO2
January, 2005	Conference 1st INTACT meeting, (Vienna, Austria)	Research	Europe	50	all partners
November, 2004	Press “24 horas”	general	Peru	$>10^5$	CNIO2
November, 2004	Press release “agencia EFE”	general	Spain South America	$>10^6$	CNIO2
November, 2004	Press “El Universal-Caracas”	general	Peru	$>10^5$	CNIO2

Third Annual Periodic Activity Report (2006)

November, 2004	Press “Diario Medico”	general research	Spain	$>10^5$	CNIO2
November, 2004	Press “Diario hoy”	general	Argentina	$>10^5$	CNIO2
November, 2004	Press “BBC mundo”	general	Spain South America	$>10^6$	CNIO2
November, 2004	Press “BBC News World Edition”	general	World wide	$>10^6$	CNIO2
November, 2004	Press release “National Mexican press agency”	general	Mexico	$>10^5$	CNIO2
November, 2004	Press “El Universal”	general	Mexico	$>10^5$	CNIO2
November, 2004	Press “Gobierno en Línea”	general	Venezuela	$>10^5$	CNIO2
November, 2004	Press Nat Rev Cancer 2004, 4:923	research	World wide	$>10^5$	CNIO2
November, 2004	Internet “Hispa-mp3”	general	South America	$>10^6$	CNIO2
November, 2004	Press “Morgenwelt”	general	Germany	$>10^6$	CNIO2
November, 2004	Press “El Siglo”	general	Mexico	$>10^5$	CNIO2

Section 3 – Publishable results

WP20 has so far not given rise to exploitable results.

Partner 4, GEM (WP 18)

Section 1 – Exploitable knowledge at its use (month 36)

None of the results from the WP have lead to exploitable results or patents

Section 2 -Dissemination of knowledge (month 36)

Publications (months 1-36)

2006

1. Rubiolo, C., Piazzolla, D., Meissl, K., Beug, H., Huber, J. C., Kolbus, A., and **Baccarini, M.** (2006) A balance between Raf-1 and Fas expression sets the pace of erythroid differentiation. *Blood* 108, 152-159
2. Galabova-Kovacs, G., Kolbus, A., Matzen, D., Meissl, K., Piazzolla, D., Rubiolo, C., Steinitz, K., and **Baccarini, M.** (2006) ERK and beyond: insights from B-Raf and Raf-1 conditional knockouts. *Cell Cycle* 5, 1514-1518
3. Galabova-Kovacs, G., Matzen, D., Piazzolla, D., Meissl, K., Plyushch, T., Chen, A. P., Silva, A., and **Baccarini, M.** (2006) Essential role of B-Raf in ERK activation during extraembryonic development. *Proc Natl Acad Sci U S A* 103, 1325-1330

2005

4. Piazzolla, D., Meissl, K., Kucerova, L., Rubiolo, C., and **Baccarini, M.** (2005) Raf-1 sets the threshold of Fas sensitivity by modulating Rok- α signaling. *J. Cell Biol.* 171, 1013-1022
5. **Baccarini, M.** (2005) Second nature: Biological functions of the Raf-1 "kinase". *FEBS Lett* 579, 3271-3277
6. Ehrenreiter, K., Piazzolla, D., Velamoor, V., Sobczak, I., Small, J. V., Takeda, J., Leung, T., and **Baccarini, M.** (2005) Raf-1 regulates Rho signaling and cell migration. *J Cell Biol* 168, 955-964

2004

7. O'Neill, E., Rushworth, L., **Baccarini, M.**, and Kolch, W. (2004) Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. *Science* 306, 2267-2270

Third Annual Periodic Activity Report (2006)

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
January, 2006	2 nd INTACT meeting, Madrid	Research	Consortium members/SAB	50	all partners
June, 2006	Lecture, CNRS Nice, France	Research	Worldwide	>50	GEM
September, 2006	XXXI st Symposium on Hormones and Regulation: Cancer Cell Signalling, Mont Saint-Odile	Research	Worldwide	>10 ²	GEM
September, 2006	ECDO's 14 th Euroconference on Apoptosis, Cagliari	Research	Worldwide	>10 ²	GEM
October, 2006	14 th International Conference of the International Society of Differentiation, Innsbruck	Research	Worldwide	>10 ²	GEM
October, 2006	Lecture, Institute of Pharmacology, Medical university of Vienna	Research	Worldwide	>50	GEM
December, 2006	Lecture, Ludwig Institute for Cancer Research, London, UK	Research	Worldwide	20	GEM
January, 2005	1 st INTACT meeting, Vienna, Austria	Research	Consortium members/SAB	50	all partners
March, 2005	Meeting on Tumor Invasion and Metastasis, Vienna, Austria	Research	Worldwide	>10 ²	GEM
July, 2005	30 th FEBS Congress - 9 th IUBMB Conference, Budapest	Research	Worldwide	>10 ²	GEM
October, 2005	13 th Euroconference on Apoptosis, Budapest	Research	Worldwide	>10 ²	GEM
October, 2005	Lecture, IFOM-IEO, Milan, Italy	Research	Worldwide	>50	GEM
November, 2005	Lecture, International PhD Research Training Programme "Transcriptional Control in Developmental Processes", Marburg, Germany	Research	Worldwide	30	GEM
September 2004	12 th Euroconference on Apoptosis, Chania, Greece	Research	Worldwide	>10 ²	GEM

Section 3 – Publishable results

WP 18 has so far not given rise to exploitable results.

Partner 5, MDC (WP 25)

Section 1 – Exploitable knowledge and its use

In the first year, none of the research undertaken has led to exploitable results or patents.

Likewise, in the year two, none of the research undertaken has led to exploitable results or patents.

Section 2 - Dissemination of knowledge (month 36)

1. Braig, M. and C.A. Schmitt. 2006. Oncogene-induced senescence: putting the brakes on tumor development. *Cancer Res.* 66: 2881-2884.
2. Schmitt, C.A. 2007. Cellular senescence and cancer treatment. *Biochim. Biophys. Acta* 1775:5-20. Epub 2006 Aug 25.
3. Reimann, M., C. Loddenkemper, C. Rudolph, I. Schildhauer, B. Teichmann, H. Stein, B. Schlegelberger, B. Dörken, and C.A. Schmitt. Myc-evoked oxidative stress selects against an Atm-governed DNA damage response predetermining treatment resistance in primary lymphomas in vivo. Under revision.
4. Braig, M., S. Lee, C. Loddenkemper, C. Rudolph, A.H.F.M. Peters, B. Schlegelberger, H. Stein, B. Dörken, T. Jenuwein and C.A. Schmitt. 2005. Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* 436: 660-665
5. Kahlem, P., B. Dörken and C.A. Schmitt. 2004. Cellular senescence in cancer treatment: friend or foe? *J Clin Invest.* 113: 169-174

Actual dates	type	Type of audience	Countries addressed	Size of audience	Partner involved
01/2006, Berlin	Conference	Hematology/Oncology	Germany	200	MDC
01/2006, Garmisch	Conference	Hematology/Oncology	EU	200	MDC
02/2006, Ulm	Seminar	Hematology/Oncology	EU	100	MDC
02/2006, Berlin	Conference	Mol. Biol.	USA, EU, Asia	150	MDC
02/2006, Berlin	Seminar	Mol. Biol.	Germany	30	MDC
02/2006, Berlin	Conference	Hematology/Oncology	USA, EU, Asia	700	MDC
02/2006, Berlin	Conference	Mol. Biol.	USA, EU, Asia	200	MDC
04/2006, USA	Conference	Mol. Biol.	USA, EU, Asia	1000	MDC
04/2006, USA	Conference	Mol. Biol.	USA, EU, Asia	300	MDC
05/2006, Essen	Seminar	Hematology/Oncology	EU	30	MDC
05/2006, Marburg	Seminar	Mol. Biol.	EU	30	MDC, UMAR
07/2006, Milan	Conference	Mol. Biol.	USA, EU, Asia	50	MDC
07/2006, Milan	Seminar	Mol. Biol.	USA, EU, Asia	200	MDC
09/2006, York	Conference	Mol. Biol.	USA, EU, Asia	300	MDC
10/2006, Cascais	Conference	Hematology/Oncology	USA, EU, Asia	400	MDC
10/2006, Borstel	Seminar	Mol. Biol.	EU, Asia	30	MDC
11/2006, Leipzig	Conference	Hematology/Oncology	USA, EU, Asia	200	MDC
11/2006, Leipzig	Conference	Hematology/Oncology	USA, EU, Asia	200	MDC
11/2006, Leipzig	Conference	Hematology/Oncology	USA, EU, Asia	200	MDC
12/2006, USA	Conference	Hematology/Oncology	USA, EU, Asia	400	MDC
04/2005, USA	Conference	Mol. Oncol.	USA, EU, Asia	10.000	MDC, NKI1, NKI2
04/2005, York (UK)	Conference	Mol. Biol.	EU	200	MDC
04/2005, Hannover	Seminar	Mol. Biol.	EU	30	MDC
05/2005, Marburg	Seminar	Mol. Biol.	EU	50	MDC, UMAR
06/2005, Falsterbo	Conference	Mol. Biol.	EU, Asia	100	MDC
10/2005 Hannover	Conference	Hematology/Oncology	USA, EU	3000	MDC
11/2005 Greifswald	Seminar	Hematology and Mol.	Germany	100	MDC

Third Annual Periodic Activity Report (2006)

		Biol.			
12/2005 USA	Conference	Hematology/Oncology	USA, EU, Asia	7.000	MDC
1/2004, Berlin	Conference	Oncology	Germany	200	MDC
1/2004, Ulm	Seminar	Oncology	EU, Asia	50	MDC
2/2004, Berlin	Conference	Oncology	EU	300	MDC
3/2004, Stockholm	Seminar	Mol. Biol.	EU	200	MDC
3/2004, Manchester	Seminar	Mol. Biol.	EU, Asia	250	MDC
4/2004, Cadenabbia	Conference	Mol. Biol.	USA,EU,Asia	50	MDC
8/2004, USA	Conference	Genetics	USA,EU,Asia	450	MDC
10/2004, Innsbruck	Conference	Hematology/Oncology	USA, EU	3000	MDC
10/2004, Hamburg	Seminar	Mol. Biol.	EU, Asia	50	MDC
11/2004, Malmö	Seminar	Mol. Biol.	EU	50	MDC
11/2004, Munich	Seminar	Mol. Biol.	EU	50	MDC
12/2004, Würzburg	Conference	Mol. Biol.	EU, USA	100	MDC
12/2004, Amsterdam	Conference	Oncology	EU, Asia	100	MDC, NK11, NK12
12/2004, USA	Conference	Hematology/Oncology	USA, EU	5.000	MDC

Section 3 – Exploitable results

This workpackage has so far not given rise to exploitable results

Partner 6, UNIMAR (WP 21, 22)

Section 1 – Exploitable knowledge at its use

Patent Protection for one of the results of the RNAi Screens (Synthetic Lethal Screen in Neuroblastoma) is being sought.

Section 2 -Dissemination of knowledge (month 36)

2006

1. Schlee, M., Hölzel, M., Bernard, S., Mailhammer, R., Schuhmacher, M., Reschke, J., Eick, D., Marinkovic, D., Wirth, T., Rosenwald, A., Staudt, L.M., Eilers, M., Baran-Marszak, F., Fagard, R., Feuillard, J., Laux, G., and Bornkamm, G.W. (2006) c-MYC activation impairs the NF- κ B and the interferon response: implications for the pathogenesis of Burkitt's lymphoma *Int. J. Cancer*, in the press
2. Fulda, S. ; Proemba, C., Berwanger, B., Häcker, S., Berthold, F., *Eilers, M.*, Christiansen, H., Hero, B., and Debatin, K.M. (2006) Loss of caspase-8 expression does not correlate with *MYCN* amplification, aggressive disease or prognosis in neuroblastoma *Cancer Research* 65, 10016-23.
3. Ritter, M., Kattmann, D., Hartman, O., Meyer, M., Burchert, A., Kim, T., Rieder, H., Wilke, A., Thiede, C., Ehninger, E., Ueki, N., Hayman, M., Schäfer, H., **Eilers, M.**, Neubauer, A. (2006) Repression of retinoic acid receptor signaling by SKI in prognostically unfavorable forms of AML *Leukemia* 20(3):437-43
4. Gebhardt, A., Frye, M., Herold, S., Aznar-Benitah, S., Braun, K., Samans, B., Watt, F., Elsässer, H.P. and **Eilers, M.** (2006) Myc regulates keratinocyte adhesion and differentiation via complex formation with Miz1 *J. Cell Biology*, 2006 172(1):139-49

2005

5. Adhikary, S. and **Eilers, M.** Transcriptional Regulation and Transformation by Myc Proteins *Nature Reviews Molecular Biology*, (2005) 6, 635-645
6. Storre J, Schafer A, Reichert N, Barbero JL, Hauser S, **Eilers M**, Gaubatz S. (2005) Silencing of the meiotic genes SMC1beta and STAG3 in somatic cells by E2F6. *J Biol Chem*. 280(50):41380-6.
7. Zirn, B. , Hartmann, O., Samans, B., Kraus, M., Filmer, A., Wittmann, S., Graf, N. , **Eilers, M.** , Gessler, M. (2005) Expression profiling of Wilms tumors reveals novel prognostic markers. *International Journal of Cancer*, Nov 14; [Epub ahead of print]
8. Adhikary, S., Marinoni, F., Hock, A., Beier, R., Bernard, S., Quarto, M., Capra, M., Goettig, S., Kogel, U., Scheffner, M., Helin, K. and **Eilers, M.** (2005) The E3 ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation *Cell* 123, 409-423.
9. Zirn, B., Samans, B., Spangenberg, C. , Graf, N. , **Eilers, M.**, Gessler, M. (2005) All-trans retinoic acid treatment of Wilms tumour cells reverses expression of genes associated with high risk and relapse in vivo *Oncogene*. 2005 May 9; [Epub ahead of print].
10. Kruetzfeldt, M.; Ellis, M., Weekes, D., Bull, J., **Eilers, M.** Vivanco, M., Sellers, W, Mitnacht, S. (2005) Selective ablation of retinoblastoma protein function by the RET finger protein *Molecular Cell* 18, 231-224.

11. Ceballos, E., Muñoz-Alonso, M., Berwanger, B., Hernández, R., Krause, M., Hartmann, O., **Eilers, M.** and Javier León*1 (2005) Inhibitory effect of c-Myc on p53-induced apoptosis in leukemia cells. Microarray analysis reveals defective induction of p53 target genes and up-regulation of chaperone genes *Oncogene*, 24, 4559-4571.
12. Rottmann, S., Menkel, A., Bouchard, C., Mertsching, J., Loidl, P., Kremmer, E., **Eilers, M.**, Lüscher-Firzlaff, J., Lilischkis, R., and Lüscher, B. (2005) Mad1 function in cell proliferation and transcriptional repression is antagonized by cyclin E/Cdk2 *Journal of Biological Chemistry* 280, 15489-92.
13. Etard, C., Gradl, D., **Eilers, M.** and Wedlich, D. (2005) Pontin and Reptin regulate cell proliferation in early *Xenopus* embryos in collaboration with c-Myc and Miz-1 *Mechanisms of Development* 122, 545-566.
14. Wanzel, M., Kleine-Kohlbrecher, D., Herold, S., Hock, A., Berns, K., Park, J., Hemmings, B. and **Eilers, M.** (2005) Akt and 14-3-3 regulate Miz1 to control cell cycle arrest upon DNA damage *Nat Cell Biol.* 7, 30-41.

2004

15. Bouchard, C., Marquardt, J., Bras, A., Medema, R. and **Eilers, M.** (2004) Requirement for PKB/Akt-mediated phosphorylation of FoxO proteins in Myc-induced proliferation and transformation. *EMBO J.* 23, 2830-2840.
16. Burchert, A., Cai, D., Hofbauer, L., Samuelsson, M., Slater, E., Duyster, J., Ritter, M., Hochhaus, A., Müller, R., **Eilers, M.**, Schmid, M., and Neubauer, A: (2004) Interferon Consensus Binding Protein (ICSBP, IRF-8) antagonizes BCR/ABL and down-regulates bcl-2 *Blood*, 103(9):3480-9.

ACTUAL DATES	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
25.1.-27.1.06, Madrid, Spain, Europe	Meeting	Oncology	European	50	All INTACT Members
27.01.-31.01.06, Santander, Spain, Europe	Seminar	Oncology	Spanish	150	Universität Marburg
28.02.- 01.03.06, Glasgow, UK, Europe	Meeting	Oncology	Europe	200	Universität Marburg
7.-12.3.06, San Francisco, USA	Seminar	Oncology	Europe, USA	100	Universität Marburg
20.03.- 22.3.06, Kleinwalsertal, Germany, Europe	Meeting	Oncology	German	100	Universität Marburg
24.-25.03.06, Heidelberg, Germany, Europe	Meeting	Oncology	German	75	Universität Marburg
9.5.06, Tübingen, Germany, Europe	Seminar	Oncology	International	300	Universität Marburg
16.5.-17.5.06, London, UK, Europe	Seminar	Oncology	International	100	Universität Marburg
14.06.06, Darmstadt, Germany, Europe	Seminar	Oncology	International	50	Universität Marburg
22.06.06, Berlin, Germany, Europe	Seminar	Oncology	Europe	75	Universität Marburg
27.06.06, Würzburg, Germany, Europe	Meeting	Oncology	Europe	200	Universität Marburg
16.-20.08.06, New York, USA	Meeting	Oncology	International	400	Universität Marburg BRIC
21.-23.09.06, Heidelberg, Germany, Europe	Meeting	Oncology	Europe	75	Universität Marburg
25.-26.09.06, Berlin, Germany, Europe	Meeting	Oncology	Europe	75	Universität Marburg

Third Annual Periodic Activity Report (2006)

11.-14.10.06, Lausanne, Switzerland, Europe	Meeting	Oncology	International	400	Universität Marburg ISREC
19.10.06, Utrecht, Netherlands, Europe	Seminar	Oncology	Europe	150	Universität Marburg
07.11.06, Leipzig, Germany, Europe	Seminar	Oncology	International	500	Universität Marburg
13.-15.11.06, London, UK, Europe	Seminar	Oncology	Europe	2 x 100	Universität Marburg
20.11.06, Wien, Austria, Europe	Meeting	Oncology	Europe	75	Universität Marburg
07.12.06, Jena, Germany, Europe	Seminar	Oncology	German	100	Universität Marburg
11. - 13.12.06, Warwick, UK, Europe	Meeting	Oncology	Europe	200	Universität Marburg NKI
17.11.05, Wien	Conference	Oncology	EU	200	UNIMAR
24.09.05, Tübingen	Meeting	Oncology	EU	50	UNIMAR
15.09.05, Jena	Meeting	Oncology	EU	50	UNIMAR
7.07.05, Stanford	Seminar	Oncology	USA	200	UNIMAR
6.07.05, San Francisco	Seminar	Oncology	USA	50	UNIMAR
20.3.05, Seattle	Conference	Oncology	USA	300	UNIMAR
14.12.04	Meeting	Oncology	EU	50	UNIMAR
10.11.04, Erlangen	Meeting	Oncology	EU	50	UNIMAR
25.10.04, Innsbruck	Workshop	Oncology	EU	50	UNIMAR
19.10.04, Tübingen	Workshop	Oncology	EU	50	UNIMAR
23.09.04, Wiesbaden	Meeting	Oncology	USA, EU	100	UNIMAR
14.06.04, Berlin	Meeting	Oncology	EU	50	UNIMAR
14.06.04, Heidelberg	Conference	Oncology	EU	200	UNIMAR
14.06.04, Hannover	Seminar	Oncology	EU	50	UNIMAR
12.02.04, Ulm	Meeting	Oncology	EU	50	UNIMAR

Section 3 – Publishable results

This workpackage has so far not given rise to exploitable results.

Partner 7, ISREC1 (WP23)

Section 1 – Exploitable knowledge and its use (month 36)

In the first year, none of the research undertaken has led to exploitable results or patents.

Likewise, in the year two, none of the research undertaken has led to exploitable results or patents.

Section 2 - Dissemination of knowledge (month 36)

2006

1. Beermann F., Kaloulis K., Hofmann D., Murisier F., Bucher P. and Trumpp A. (2006). Identification of evolutionarily conserved regulatory elements in the mouse Fgf8 locus. *Genesis* 44(1):1-6
2. Prathapam T., Tegen S., Oskarsson T., Trumpp A. and Martin G.S (2006). Activated Src abrogates the Myc requirement for the G0/G1 transition but not for the G1/S transition. *Proc Natl Acad Sci USA*, in press
3. Bianchi,T, Gasser, S., Trumpp, A. and MacDonald, H.R. (2006). c-Myc acts downstream of IL-15 in the regulation of memory CD8 T cell homeostasis. *Blood*, Jan 31; [Epub ahead of print] PMID: 16449532
4. Oskarsson T., Essers M., Dubois D., Dubey C., Roger C., Metzger D., Chambon P., Hummler E., Beard P., and Trumpp A. (2006). Skin epidermis lacking the *c-myc* gene is resistant to Ras driven tumorigenesis but can re-acquire sensitivity upon additional loss of the p21^{CIP1} gene. *GenesDev* 20: 2024-2029.
5. Wilson A. and Trumpp A. (2006). The bone marrow stem cell niche. *Nature Reviews Immunology*, 6:93-106

2005

6. Yosef Refaeli, Kenneth A. Field, Andreas Trumpp and J. Michael Bishop (2005). The Proto-oncogene MYC can Break B-Cell Tolerance. *Proc Natl Acad Sci USA*. Mar 15;102(11):4097-102
7. Ackermann J., Fruttschi M., Kaloulis K., McKee T., Trumpp A. and Beermann F. (2005) Metastasizing melanoma formation caused by expression of activated N-rasQ61K on an INK4a-deficient background. *Cancer Research* 65 (10): 4005-4011
8. Michael D. Bettess, Mark J. Murphy, Christelle Dubey, Nicole Dubois, Catherine Roger, Sylvie Robine and Andreas Trumpp (2005). c-Myc is required for the formation of intestinal crypts, but dispensable for homeostasis of the adult intestinal epithelium. *Molecular and Cellular Biology* 25 (17): 7868-7878.
9. Riggi N., Cironi P.P., Suva M.-L., Kaloulis K., Garcia-Echeveria C., Hoffmann F., Trumpp A. and Stamenkovic I. (2005). The EWS-FLI fusion protein transforms primary mesenchymal progenitor cells to form tumors with molecular and morphological features of Ewing's sarcoma. *Cancer Research* 65 (24):11459-68
10. Murphy M.J., Wilson A. and Trumpp A. (2005). More than proliferation: Myc function in stem cells. *Trends in Cell Biology*, 15(3):128-137
11. Oskarsson T. and Trumpp A. (2005). The Myc trilogy: Lord of RNA Polymerases. *Nat Cell Biol.* 2005 Mar;7(3):215-7.

2004

Third Annual Periodic Activity Report (2006)

12. Wilson A., Murphy M. , Kaloulis K., Bettess M., Pasche A.C., MacDonald H.R. and Trumpp A., (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes&Dev* 18;2747-2763. This article was picked and evaluated by one "Faculty of 1000" member and has received a F1000 Score of 3.0.

13. Trumpp A and Kalman D. (2004). Book Review: J.M. Bishop: How to win the Nobel Prize. *Nat Cell Biol.* 6: 173

ACTUAL DATES	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
Nov.15-17, 2006; Berlin, Germany	Seminar	Oncology	EU	200	ISREC1
Nov. 13-15 2006, Ottawa, Canada.	Seminar	Biology	Canada	200	ISREC1
Oct. 6-8, 2006, Strasbourg	Seminar	Biology	EU	200	ISREC1
Oct. 5-7, 2006, Germany	Seminar	Oncology	EU	200	ISREC1
Sept. 14-16, 2006, Tübingen, Germany	Seminar	Biology	EU	200	ISREC1
Sept. 11-13, 2006, Lausanne	Seminar	Biology	EU	200	ISREC1
Mar 12-14, 2006, Heidelberg, Germany	Seminar	Oncology	EU	200	ISREC1
November 10-12, 2005, Milan	Conference	Oncology	EU	200	ISREC1
October 19-23, 2005, Lausanne	Conference	Biology	EU	200	ISREC1
September 30. 2005, Lund	Seminar	Biology	EU	200	ISREC1
August 25-26. 2005, Winter Park	Workshop	Biology	USA	200	ISREC1
June 29. 2005, Vancouver	Seminar	Oncology	Canada	200	ISREC1
June 28. 2005, Seattle	Seminar	Oncology	USA	50	ISREC1
June 23-25. 2005, San Francisco	Conference	Oncology	USA	300	ISREC1
June 19-22. 2005, Glasgow	Conference	Oncology	EU	200	ISREC1
April 15-18. 2005, Cascais, Portugal	Seminar	Oncology	EU	250	ISREC1
2004 Utrecht Netherlands	Lecturer at the MASERCLASS	Oncology	EU	50	ISREC1
September 25-27. 2004, Barolo, Italy	Workshop	Oncology	EU	200	ISREC1
2004 29. July-11. August, Banbury Cold Spring Harbor	Course lecturer	Oncology	USA, EU	100	ISREC1
April 22-24, 2004, Heidelberg Germany	Symposium	Oncology	EU	200	ISREC1
April 17-20 2004, Heidelberg, Germany	Conference	Oncology	EU/USA	200	ISREC1
February 2004, Fribourg, Switzerland	Conference	Oncology	EU	200	ISREC1
19. Jan. 2004, Berne, Switzerland	Seminar	Oncology	EU, USA	50	ISREC1

Section 3 – Exploitable results

This workpackage has so far not given rise to exploitable results

Partner 7, ISREC2 (WP 6)

Section 1 – Exploitable knowledge at its use (month 36)

None of the results from the WP has lead to exploitable results or patents

Section 2 - Dissemination of knowledge (month 36)

Publications (months 1-36)

2006

Wang X.D., Leow, C.C., Zha, J., Tang, Z., Modrusan, Z., Radtke, F., Aguet, M., de Sauvage, F.J., and Gao, W.Q. (2006). Notch signaling is required for normal prostatic epithelial cell proliferation and differentiation. *Dev Biol* **290**, 66-80.

2005

Sperisen, P., Schmid, C. D., Bucher, P. , and Zilian, O. (2005). Stealth proteins: in silico identification of a novel protein family rendering bacterial pathogens invisible to host immunedefense. *PLoS Comput Biol* **1**, e63.

2004

Klein, A. L., Zilian, O., Suter, U. and Taylor, V. (2004). Murine numb regulates granule cell maturation in the cerebellum. *Dev Biol* **266**, 161-177.

DATE	TYPE	TYPE OF AUDIENC E	COUNTRIES ADDRESSED	SIZE OF AUDIENC E	PARTNER INVOLVE D
April 2006	Seminar, Keystone Symposia: Wnt and β -Catenin Signaling in Development and Disease, Snowbird, Utah, USA	Research	World wide	$>10^2$	ISREC2
September 2006	Seminar, ISREC, Switzerland	Research	Switzerland	$>10^2$	ISREC2
November 2005	Seminar, ISREC, Switzerland	Research	Switzerland	$>10^2$	ISREC2

Section 3 – Publishable results

WP6 has so far not given rise to exploitable results.

Partner 8, BICR (WP 10, 11)

Section 1 – Exploitable knowledge at its use (month 36)

None of the results from the WPs have lead to exploitable results or patents

Section 2 -Dissemination of knowledge (month 36)

Publications (months 1-36)

2006

1. Uldrijan S, Pannekoek WJ, **Vousden KH**. An essential function of the extreme C-terminus of MDM2 can be provided by MDMX. EMBO J. 2007 Jan 10;26(1):102-12.
2. **Vousden KH**. Outcomes of p53 activation - spoilt for choice. J Cell Sci. 2006 Dec 15;119(Pt 24):5015-20.
3. Wilson JM, Henderson G, Black F, Sutherland A, Ludwig RL, **Vousden KH**, RobinsDJ. Synthesis of 5-deazaflavin derivatives and their activation of p53 in cells. Bioorg Med Chem. 2007 Jan 1;15(1):77-86.
4. Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, **Vousden KH**. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell. 2006 Jul 14;126(1):107-20.
5. Davydov IV, Woods D, Safiran YJ, Oberoi P, Fearnhead HO, Fang S, Jensen JP, Weissman AM, Kenten JH, **Vousden KH**. Assay for ubiquitin ligase activity: high-throughput screen for inhibitors of HDM2. J Biomol Screen. 2004 Dec;9(8):695-703.

2005

1. Bensaad K, **Vousden KH**. Savior and slayer: the two faces of p53. Nat Med. 2005 Dec;11(12):1278-9.
2. **Vousden KH**. Apoptosis. p53 and PUMA: a deadly duo. Science. 2005 Sep 9;309(5741):1685-6.
3. Yang Y, Ludwig RL, Jensen JP, Pierre SA, Medaglia MV, Davydov IV, Safiran YJ, Oberoi P, Kenten JH, Phillips AC, Weissman AM, **Vousden KH**. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. Cancer Cell. 2005 Jun;7(6):547-59.
4. Yee KS, **Vousden KH**. Complicating the complexity of p53. Carcinogenesis. 2005 Aug;26(8):1317-22.
5. Fogal V, Kartasheva NN, Trigiante G, Llanos S, Yap D, **Vousden KH**, Lu X. ASPP1 and ASPP2 are new transcriptional targets of E2F. Cell Death Differ. 2005 Apr;12(4):369-76.
6. Weber HO, Ludwig RL, Morrison D, Kotlyarov A, Gaestel M, **Vousden KH**. HDM2 phosphorylation by MAPKAP kinase 2. Oncogene. 2005 Mar 17;24(12):1965-72.
7. Rossi M, De Laurenzi V, Munarriz E, Green DR, Liu YC, **Vousden KH**, Cesareni G, Melino G. The ubiquitin-protein ligase Itch regulates p73 stability. EMBO J. 2005 Feb 23;24(4):836-48.
8. **Vousden KH**, Prives C. p53 and prognosis: new insights and further complexity. Cell. 2005 Jan 14;120(1):7-10.

2004

Third Annual Periodic Activity Report (2006)

9. Davydov IV, Woods D, Safiran YJ, Oberoi P, Fearnhead HO, Fang S, Jensen JP, Weissman AM, Kenten JH, **Vousden KH**. Assay for ubiquitin ligase activity: high-throughput screen for inhibitors of HDM2. *J Biomol Screen*. 2004 Dec;9(8):695-703.
10. Ryan KM, O'Prey J, **Vousden KH**. Loss of nuclear factor-kappaB is tumor promoting but does not substitute for loss of p53. *Cancer Res*. 2004 Jul 1;64(13):4415-8.
11. Bernardi R, Scaglioni PP, Bergmann S, Horn HF, **Vousden KH**, Pandolfi PP. PML regulates p53 stability by sequestering Mdm2 to the nucleolus. *Nat Cell Biol*. 2004 Jul;6(7):665-72.
12. Terrinoni A, Ranalli M, Cadot B, Leta A, Bagetta G, **Vousden KH**, Melino G. p73-alpha is capable of inducing scotin and ER stress. *Oncogene*. 2004 Apr 29;23(20):3721-5.
13. Horn HF, **Vousden KH**. Cancer: guarding the guardian? *Nature*. 2004 Jan 8;427(6970):110-1.
14. Melino G, Bernassola F, Ranalli M, Yee K, Zong WX, Corazzari M, Knight RA, Green DR, Thompson C, **Vousden KH**. p73 Induces apoptosis via PUMA transactivation and Bax mitochondrial translocation. *J Biol Chem*. 2004 Feb 27;279(9):8076-83.

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
January, 2006	Second Annual INTACT meeting (Madrid, Spain)	Research	World wide	50	all partners
January, 2005	First Annual INTACT meeting (Vienna, Austria)	Research	World wide	50	all partners
December, 2005	ICRASH summit, (Amsterdam, Netherlands)	Research	Worldwide	20	BICR

Section 3 – Publishable results

WP10 and WP11 have so far not given rise to exploitable results.

Partner 9, Pasteur (WP 19)

Section 1 – Exploitable knowledge at its use (month 36)

None of the results from the WP have lead to exploitable results or patents

Section 2 -Dissemination of knowledge (month 36)

Publications (months 1-36)

2006

1. Bischof, O., Schwamborn, K., Martin, N., Werner, A., , Sustmann, C., Grosschedl, R. and **Dejean, A.** The E3 SUMO Ligase PIASy is a Novel Regulator of Cellular Senescence and Apoptosis. *Mol. Cell*, 2006, 22, 783-94.
2. Kumar, P.*, Bischof, O.*, Kumar Purbey, P., Notani, D., Urlaub, H., **Dejean, A.**, and Galande, S. Functional Interaction between PML and SATB1 Regulates Chromatin Loop Architecture and Transcription of the MHC class I Locus (* first co-authors). *Nature Cell. Biol.*, in press.
3. Xhemalce, B., Riising, E.M., Baumann, P., **Dejean, A.**, Arcangioli, B. and Seeler, JS. Role of SUMO in the Dynamics of Telomere Maintenance in Fission Yeast. *Proc. Natl. Acad. Sci. USA*, in press.

2005

4. Bischof, O., Nacerddine, K. and **Dejean, A.** HPV oncoprotein E7 targets PML and circumvents cellular senescence via the Rb and p53 tumor suppressor pathways. *Mol. Cell. Biol.*, 2005, 25, 1013-1024.
5. Van den Akker, E., Ano, S., Shih, H.M., Wang, L.C., Pironin, M., Palvimo, J.J., Kotaja, N., Kirsh, O., **Dejean, A.** and Ghysdael, J. FLI-1 functionally interacts with PIASXalpha, a member of the PIAS E3-SUMO-ligase family. *J. Biol. Chem.*, 2005, 280, 38035-46.
6. Nacerddine, K., Lehembre, F., Bhaumik, M. Artus, J., Cohen-Tanoudji, M., Babinet, C., Pandolfi, P.P. and **Dejean, A.** The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice, *Dev. Cell*, 2005, 9, 769-779.

2004

7. Xhemalce, B.,* Seeler, J-S.,* Thon, G., **Dejean, A.**, and Arcangioli, B. Role of the Fission Yeast SUMO E3 Ligase Pli1p in Centromere and Telomere Maintenance. *EMBO J*, 2004, 23, 3844-53. (* first co-authors)
8. Chauchereau, A., Mathieu, M., de Saintignon, J., Ferreira, R., Pritchard, L., Mishal, Z., **Dejean, A.** and Harel-Bellan A. HDAC4 mediates transcriptional repression by acute promyelocytic leukemia-associated protein PLZF. *Oncogene*, 2004, 23, 8777-84.

Third Annual Periodic Activity Report (2006)

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
November 2006	Seminar, Rubicon Florence (Italy)	Research	World wide	$>10^2$	Pasteur
October 2006	Lecture, BCCS Bergen (Norway)	Research	World wide	$>10^2$	Pasteur
June 2006	Seminar, INSERM Paris (France)	Research	World wide	20	Pasteur
May 2006	Lecture, MacGill Montreal (Canada)	Research	World wide	$>10^2$	Pasteur
March 2006	Seminar, Rubicon Stockholm (Sweden)	Research	World wide	30	Pasteur
February 2006	Course, Pasteur Paris (France)	Research	World wide	50	Pasteur
September 2005	Seminar, APL symposium Rome (Italy)	Research	World wide	$>10^2$	Pasteur
November 2005	Lecture, IGH Montpellier (France)	Research	World wide	$>10^2$	Pasteur
May 2005	Seminar, CHU Rouen (France)	Research	World wide	$>10^2$	Pasteur
October 2004	Lecture, Norwegian Biochem. Society, Oslo (Norway)	Research	World wide	$>10^2$	Pasteur
October 2004	Seminar, Karolinska Institute, Stockholm (SE)	Research	World wide	$>10^2$	Pasteur
September 2004	Lecture, Karolinska Institute, Stockholm (SE)	Research	World wide	$>10^2$	Pasteur
September 2004	Seminar, ENS Lyon (France)	Research	World wide	80	Pasteur
July 2004	Seminar, LesTreilles Meeting, France	Research	World wide	30	Pasteur
June 2004	Seminar, Weizmann Meeting Rehovot (Israel)	Research	World wide	$>10^3$	Pasteur
January 2004	Seminar, chromatin and cancer symposium, Milano (Italy)	Research	World wide	$>10^2$	Pasteur

Section 3 – Publishable results

WP19 has so far not given rise to exploitable results.

Partner 10, MCH (WP 28) –closed

Partner 11, Agendia (WP 27)

Section 1 – Exploitable knowledge and its use

In the third year, none of the research undertaken have led to exploitable results or patents.

Section 2 -Dissemination of knowledge

Publications 2006:

None.

Publications from the years 2004, 2005:

Glas, A.M., Kersten, M.J., Delahaye, L.J.M.J., Witteveen, A.T., Kibbelaar, R.E., Velds, A., Wessels, L.F.A., Joosten, P., Kerkhoven, R.M., Bernards, R., van Krieken, J.H.J.M., Kluin, P.M., van 't Veer, L.J., de Jong, D. (2005). Gene expression profiling in Follicular Lymphoma to assess clinical aggressiveness and to guide the choice of treatment. *Blood*, 105, 301-307.

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
July 2006	Conference, Budapest (Hungary)	Medical	World wide	300	Agendia
October 2006	Conference, Luxembourg	Research	European	100	Agendia
January 2005	Conference, Vienna (A	Research	World wide	50	Agendia

Section 3 – Exploitable results

This workpackage has not given rise to exploitable results.

Partner 12, IEO (WP 4, 13, 17)

Section 1 – Exploitable knowledge at its use

In the third year none of the research undertaken has led to exploitable results or patents.

Section 2 -Dissemination of knowledge (month 36)

Publications from the years 2004, 2005, 2006

2006

1. Berry A, Capone F, Giorgio M, **Pellicci P.G.**, de Kloet ER, Alleva E, Minghetti L, Cirulli F. Deletion of the life span determinant p66(Shc) prevents age-dependent increases in emotionality and pain sensitivity in mice. *Exp Gerontol* 2006.
2. Carbone R, Botrugno OA, Ronzoni S, Insinga A, Di Croce L, **Pellicci P.G.**, Minucci S. Recruitment of the histone methyltransferase SUV39H1 and its role in the oncogenic properties of the leukemia-associated PML-retinoic acid receptor fusion protein. *Mol Cell Biol* 26(4), 1288-96, 2006.
3. Carbone R, Marangi I, Zanardi A, Giorgetti L, Chierici E, Berlanda G, Podesta A, Fiorentini F, Bongiorno G, Piseri P, **Pellicci P.G.**, Milani P. Biocompatibility of cluster-assembled nanostructured TiO(2) with primary and cancer cells. *Biomaterials* 27(17), 3221-9, 2006.
4. Colombo E, Martinelli P, Zamponi R, Shing DC, Bonetti P, Luzi L, Volorio S, Bernard L, Pruner G, Alcalay M, **Pellicci P.G.**. Delocalization and destabilization of the Arf tumor suppressor by the leukemia-associated NPM mutant. *Cancer Res* 66(6), 3044-50, 2006.
5. Falini B, Bigerna B, Pucciarini A, Tiacci E, Mecucci C, Morris SW, Bolli N, Rosati R, Hanissian S, Ma Z, Sun Y, Colombo E, Arber DA, Pacini R, La Starza R, Galletti BV, Liso A, Martelli MP, Diverio D, **Pellicci P.G.**, Coco FL, Martelli MF. Aberrant subcellular expression of nucleophosmin and NPM-MLF1 fusion protein in acute myeloid leukaemia carrying t(3;5): a comparison with NPMc+ AML. *Leukemia* 20(2), 368-371, 2006 Erratum in: *Leukemia* 20(7), 1330, 2006. Coco, FL [corrected to Lo Coco, F]; Galletti, BV [corrected to Verducci Galletti, B].
6. Falini B, Martelli MP, Bolli N, Bonasso R, Ghia E, Pallotta MT, Diverio D, Nicoletti I, Pacini R, Tabarrini A, Verducci Galletti B, Mannucci R, Roti G, Rosati R, Specchia G, Liso A, Tiacci E, Alcalay M, Luzi L, Volorio S, Bernard L, Guarini A, Amadori S, Mandelli F, Pane F, Lo Coco F, Saglio G, **Pellicci P.G.**, Martelli MF, Mecucci C. Immunohistochemistry predicts nucleophosmin (NPM) mutations in acute myeloid leukemia. *Blood* 108(6), 1999-2005, 2006.
7. Mariano AR, Colombo E, Luzi L, Martinelli P, Volorio S, Bernard L, Meani N, Bergomas R, Alcalay M, **Pellicci P.G.**. Cytoplasmic localization of NPM in myeloid leukemias is dictated by gain-of-function mutations that create a functional nuclear export signal. *Oncogene* 25(31), 4376-80, 2006.
8. Pellegrini M, Finetti F, Petronilli V, Ulivieri C, Giusti F, Lupetti P, Giorgio M, **Pellicci P.G.**, Bernardi P, Baldari CT. p66SHC promotes T cell apoptosis by inducing mitochondrial dysfunction and impaired Ca(2+) homeostasis. *Cell Death Differ* 2006.
9. Rota M, LeCapitaine N, Hosoda T, Boni A, De Angelis A, Padin-Iruegas ME, Esposito G, Vitale S, Urbanek K, Casarsa C, Giorgio M, Luscher TF, **Pellicci P.G.**, Anversa P, Leri A, Kajstura J. Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene. *Circ Res* 99(1), 42-52, 2006.
10. Trinei M, Berniakovich I, **Pellicci P.G.**, Giorgio M. Mitochondrial DNA copy number is regulated by cellular proliferation: A role for Ras and p66(Shc). *BBA - Bioenergetics* 1757(5-6), 624-30, 2006.
11. Trubia M, Albano F, Cavazzini F, Cambrin GR, Quarta G, Fabbiano F, Ciambelli F, Magro D, Hernandez JM, Mancini M, Diverio D, **Pellicci P.G.**, Coco FL, Mecucci C, Specchia G, Rocchi M, Liso V, Castoldi G, Cuneo A. Characterization of a recurrent translocation t(2;3)(p15-22;q26) occurring in acute myeloid leukaemia. *Leukemia* 20(1), 48-54, 2006.

12. Villa R, Morey L, Raker VA, Buschbeck M, Gutierrez A, De Santis F, Corsaro M, Varas F, Bossi D, Minucci S, **Pellicci P.G.**, Di Croce L. The methyl-CpG binding protein MBD1 is required for PML-RAR $\{\alpha\}$ function. Proc Natl Acad Sci U S A 103(5), 1400-5, 2006.
13. Menini S, Amadio L, Oddi G, Ricci C, Pesce C, Pugliese F, Giorgio M, Migliaccio E, **Pellicci P.G.**, Iacobini C, Pugliese G. Deletion of p66Shc Longevity Gene Protects Against Experimental Diabetic Glomerulopathy by Preventing Diabetes-Induced Oxidative Stress. Diabetes 55(6), 1642-50, 2006.
14. Pezzicoli A, Ulivieri C, Capitani N, Ventura A, **Pellicci P.G.**, Baldari CT. Expression in T-cells of the proapoptotic protein p66SHC is controlled by promoter demethylation. Biochem Bioph Res Co 349(1), 322-8, 2006.
15. Cimino G, Lo-Coco F, Fenu S, Travaglini L, Finolezzi E, Mancini M, Nanni M, Careddu A, Fazi F, Padula F, Fiorini R, Aloe Spiriti MA, Petti MC, Venditti A, Amadori S, Mandelli F, **Pellicci P.G.**, Nervi C. Sequential Valproic Acid/All-trans Retinoic Acid Treatment Reprograms Differentiation in Refractory and High-Risk Acute Myeloid Leukemia. Cancer Res 66(17), 8903-11, 2006.
16. Marchesi F, Minucci S, **Pellicci P.G.**, Gobbi A, Scanziani E. Immunohistochemical Detection of Ym1/Ym2 Chitinase-like Lectins Associated with Hyalinosis and Polypoid Adenomas of the Transitional Epithelium in a Mouse with Acute Myeloid Leukemia. Vet Pathol 43(5), 773-6, 2006.
17. Orsini F, Moroni M, Contursi C, Yano M, **Pellicci P.G.**, Giorgio M, Migliaccio E. Regulatory effects of the mitochondrial energetic status on mitochondrial p66(Shc). Biol Chem. 387(10-11), 1405-10, 2006.
18. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, Schurra C, Garre' M, Nuciforo PG, Bensimon A, Maestro R, **Pellicci PG**, d'Adda di Fagagna F. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 444(7119), 638-42, 2006.
19. Buonaguro FM, Lewis GK, **Pellicci PG** Introducing infectious agents and cancer. Infect Agent Cancer 14,1-1, 2006.
20. Carbone R., Marangi I., Zanardi A., Giorgetti L., Chierici E., Berlanda G., Podesta' A., Fiorentini F., Bongiorno G., Piseri P., **Pellicci P.G.**, Milani P. Biocompatibility of cluster-assembled nanostructured TiO₂ with primary and cancer cells Biomaterials. 17, 3221-9, 2006.

2005

21. Belloni E, Trubia M, Gasparini P, Micucci C, Tapinassi C, Confalonieri S, Nuciforo P, Martino B, Lo-Coco F, **Pellicci PG**, Di Fiore PP. 8p11 myeloproliferative syndrome with a novel t(7;8) translocation leading to fusion of the FGFR1 and TIF1 genes. Genes Chromosomes Cancer. 42(3):320-5. 2005.
22. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, La Starza R, Diverio D, Colombo E, Santucci A, Bigerna B, Pacini R, Pucciarini A, Liso A, Vignetti M, Fazi P, Meani N, Pettrossi V, Saglio G, Mandelli F, Lo-Coco F, **Pellicci PG**, Martelli MF; GIMEMA Acute Leukemia Working Party. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 20;352(3):254-66. 2005.
23. Fazi F, Travaglini L, Carotti D, Palitti F, Diverio D, Alcalay M, McNamara S, Miller WH, Coco FL, **Pellicci PG**, Nervi C. Retinoic acid targets DNA-methyltransferases and histone deacetylases during APL blast differentiation in vitro and in vivo. Oncogene. 10;24(11):1820-30. 2005.
24. Insinga A, Monestiroli S, Ronzoni S, Gelmetti V, Marchesi F, Viale A, Altucci L, Nervi C, Minucci S, **Pellicci PG**. Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat Med. 11(2):233. 2005.
25. Insinga A, **Pellicci PG**, Inucci S. Leukemia-associated Fusion Proteins: Multiple Mechanisms of Action to Drive Cell Transformation. Cell Cycle. 19;4(1). 2005.
26. Kindle KB, Troke PJ, Collins HM, Matsuda S, Bossi D, Bellodi C, Kalkhoven E, Salomoni P, **Pellicci PG**, Minucci S, Heery DM. MOZ-TIF2 inhibits transcription by nuclear receptors and p53 by impairment of CBP function. Mol Cell Biol. 25(3):988-1002. 2005.
27. Lunghi P, Tabilio A, Lo-Coco F, **Pellicci PG**, Bonati A. Arsenic trioxide (ATO) and MEK1 inhibition synergize to induce apoptosis in acute promyelocytic leukemia cells. Leukemia. 19(2):234-44. 2005.

28. Martoriati A, Doumont G, Alcalay M, Bellefroid E, **Pellicci PG**, Marine JC. dapk1, encoding an activator of a p19ARF-p53-mediated apoptotic checkpoint, is a transcription target of p53. *Oncogene*, 17;24(8):1461-6. 2005.
29. Patrussi L, Savino MT, Pellegrini M, Paccani SR, Migliaccio E, Plyte S, Lanfranccone L, **Pellicci PG**, Baldari CT. Cooperation and selectivity of the two Grb2 binding sites of p52Shc in T-cell antigen receptor signaling to Ras family GTPases and Myc-dependent survival. *Oncogene* 24;24(13):2218-28. 2005.
30. Brenner C, Deplus R, Didelot C, Lorient A, Vire E, De Smet C, Gutierrez A, Danovi D, Bernard D, Boon T, **Pellicci PG**, Amati B, Kouzarides T, de Launoit Y, Di Croce L, Fuks F. Myc represses transcription through recruitment of DNA methyltransferase corepressor. *EMBO J.* 26;24(2):336-46. 2005.
31. Alcalay M, Tiacci E, Bergomas R, Bigerna B, Venturini E, Minardi SP, Meani N, Diverio D, Bernard L, Tizzoni L, Volorio S, Luzi L, Colombo E, Lo Coco F, Mecucci C, Falini B, **Pellicci PG**. Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc+ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance. *Blood* 1;106(3):899-902. 2005.
32. Gorletta TA, Gasparini P, D'Elia MM, Trubia M, **Pellicci PG**, Di Fiore PP. Frequent loss of heterozygosity without loss of genetic material in acute myeloid leukemia with a normal karyotype. *Genes Chromosomes Cancer* 13. 2005.
33. Graiani G, Lagrasta C, Migliaccio E, Spillmann F, Meloni M, Madeddu P, Quaini F, Padura IM, Lanfranccone L, **Pellicci PG**, Emanuelli C. Genetic Deletion of the p66Shc Adaptor Protein Protects From Angiotensin II-Induced Myocardial Damage. *Hypertension* 5. 2005.
34. Pilatino C, Cilloni D, Messa E, Morotti A, Giugliano E, Pautasso M, Familiari U, Cappia S, **Pellicci PG**, Lo Coco F, Saglio G, Guerrasio A. Increase in platelet count in older, poor-risk patients with acute myeloid leukemia or myelodysplastic syndrome treated with valproic acid and all-trans retinoic acid. *Cancer* 1;104(1):101-9. 2005.
35. Ronzoni S, Faretta M, Ballarini M, **Pellicci P**, Minucci S. New method to detect histone acetylation levels by flow cytometry. *Cytometry A*;66(1):52-61. 2005.
36. Caprodossi S, Pedinotti M, Amantini C, Santoni G, Minucci S, **Pellicci PG**, Fanelli M. Differentiation Response of Acute Promyelocytic Leukemia Cells and PML/RARa Leukemogenic Activity Studies by Real-Time RT-PCR. *Mol Biotechnol.* 30(3):231-8. 2005.
37. Insinga A, Minucci S, **Pellicci PG**. Mechanisms of selective anticancer action of histone deacetylase inhibitors. *Cell Cycle*. 4(6):741-3. 2005.
38. Meani N, Minardi S, Licciulli S, Gelmetti V, Coco FL, Nervi C, **Pellicci PG**, Muller H, Alcalay M. Molecular signature of retinoic acid treatment in acute promyelocytic leukemia. *Oncogene* 5;24(20):3358-68. 2005.
39. Lahortiga I, Vazquez I, Belloni E, Roman JP, Gasparini P, Novo FJ, Zudaire I, **Pellicci PG**, Hernandez JM, Calasanz MJ, Odero MD. FISH analysis of hematological neoplasias with 1p36 rearrangements allows the definition of a cluster of 2.5 Mb included in the minimal region deleted in 1p36 deletion syndrome. *Hum Genet.* 116(6):476-85. 2005.
40. Patrussi L, Savino MT, Pellegrini M, Paccani SR, Migliaccio E, Plyte S, Lanfranccone L, **Pellicci PG**, Baldari CT. Cooperation and selectivity of the two Grb2 binding sites of p52Shc in T-cell antigen receptor signaling to Ras family GTPases and Myc-dependent survival. *Oncogene* 24;24(13):2218-28. 2005.
41. Colombo E, Bonetti P, Lazzerini Denchi E, Martinelli P, Zamponi R, Marine JC, Helin K, Falini B, **Pellicci PG**. Nucleophosmin Is Required for DNA Integrity and p19Arf Protein Stability. *Mol Cell Biol* 25 (20):8874-86, 2005.
42. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia P, Luzi L, Minucci S, Marcaccio M, Pinton P, Rizzuto R, Bernardi P, Paolucci F, and **Pellicci PG**. Electron Transfer between Cytochrome c and p66Shc Generates Reactive Oxygen Species that Trigger Mitochondrial Apoptosis. *Cell*, Vol. 122, 1-13. 2005.

43. Belloni E., Trubia M., Mancini M., Derme V., Nanni M., Lahortiga I., Riccioni R., Confalonieri S., Lo-Coco F., Di Fiore P.P., and **Pellicci P.G.** A New Complex Rearrangement Involving the ETV6, LOC115548, and MN1 Genes in a Case of Acute Myeloid Leukemia. Genes, Chromosome & Cancer 41:272-277. 2004.
44. Danovi D, Meulmeester E, Pasini D, Migliorini D, Capra M, Frenk R, De Graaf P, Francoz S, Gasparini P, Gobbi A, Helin K, **Pellicci PG**, Jochemsen AG, Marine JC. Amplification of Mdmx (or Mdm4) Directly Contributes to Tumor Formation by Inhibiting p53 Tumor Suppressor Activity. Mol Cell Biol. 24(13):5835-43. 2004.
45. Francia P, delli Gatti C, Bachschmid M, Martin-Padura I, Savoia C, Migliaccio E, **Pellicci PG**, Schiavoni M, Luscher TF, Volpe M, Cosentino F. Deletion of p66shc gene protects against age-related endothelial dysfunction. Circulation 2;110(18):2889-95. 2004.
46. Insinga A, Monestiroli S, Ronzoni S, Carbone R, Pearson M, Pruneri G, Viale G, Appella E, **Pellicci P**, Minucci S. Impairment of p53 acetylation, stability and function by an oncogenic transcription factor. EMBO J. 10;23(5):1144-54. 2004.
47. Lahortiga I, Agirre X, Belloni E, Vazquez I, Larrayoz MJ, Gasparini P, Coco FL, **Pellicci PG**, Calasanz MJ, Odero MD. Molecular characterization of a t(1;3)(p36;q21) in a patient with MDS. MEL1 is widely expressed in normal tissues, including bone marrow, and it is not overexpressed in the t(1;3) cells. Oncogene, 23(1):311-6. 2004.
48. Mandala M, Curigliano G, Bucciarelli P, Ferretti G, Mannucci PM, Colleoni M, Ventura A, Peruzzotti G, Severi G, **Pellicci PG**, Biffi R, Orsi F, Cinieri S, Goldhirsch A. Factor V Leiden and G20210A prothrombin mutation and the risk of subclavian vein thrombosis in patients with breast cancer and a central venous catheter. Ann Oncol.; 15(4):590-3. 2004.
49. Orsini F, Migliaccio E, Moroni M, Contursi C, Raker VA, Piccini D, Martin-Padura I, Pelliccia G, Trinei M, Bono M, Puri C, Tacchetti C, Ferrini M, Mannucci R, Nicoletti I, Lanfrancone L, Giorgio M, **Pellicci PG**. The Life Span Determinant p66Shc Localizes to Mitochondria Where It Associates with Mitochondrial Heat Shock Protein 70 and Regulates Trans-membrane Potential. J Biol Chem., 11;279(24):25689-95. 2004.
50. Pacini S, Pellegrini M, Migliaccio E, Patrussi L, Ulivieri C, Ventura A, Carraro F, Naldini A, Lanfrancone L, **Pellicci P**, Baldari CT. p66SHC Promotes Apoptosis and Antagonizes Mitogenic Signaling in T Cells. Mol Cell Biol. 24(4):1747-57. 2004.
51. Transidico P., Bianchi M., Capra M., **Pellicci P.G.** and Faretta M. From Cells to Tissues: Fluorescence Confocal Microscopy in the Study of Histological Samples. Microscopy Res. And Technique, 64; 89-95. 2004.
52. Troglio F, Echart C, Gobbi A, Pawson T, **Pellicci PG**, De Simoni MG, Pelicci G. The Rai (Shc C) adaptor protein regulates the neuronal stress response and protects against cerebral ischemia. Proc Natl Acad Sci U S A. 26;101(43):15476-81. 2004.
53. Ventura A, Maccarana M, Raker VA, **Pellicci PG**. A cryptic targeting signal induces isoform-specific localization of p46Shc to mitochondria. J Biol Chem., 276:2299-2306. 2004.
54. Villa R, De Santis F, Gutierrez A, Minucci S, **Pellicci PG**, Di Croce L. Epigenetic gene silencing in acute promyelocytic leukemia. Biochem Pharmacol. 68(6):1247-54. 2004.
55. Zaccagnini G, Martelli F, Fasanaro P, Magenta A, Gaetano C, Di Carlo A, Biglioli P, Giorgio M, Martin-Padura I, **Pellicci PG**, Capogrossi MC. p66ShcA modulates tissue response to hindlimb ischemia. Circulation, 109(23):2917-23. 2004.

DATE	TYPE	TYPE OF AUDIENCE	COUNTRIES ADDRESSED	SIZE OF AUDIENCE	PARTNER INVOLVED
18-22 February 2006	ACUTE LEUKEMIAS XI – Munich (Germany)	Research	World wide	>10 ²	IEO
10 march 2006	MEETING FOR RESEARCH UNDER THE 7 TH FRAMEWORK PROGRAMME – Brussels	Research	World wide	>10 ²	IEO

Third Annual Periodic Activity Report (2006)

	(Belgium)				
16-18 March 2006	CNIO/Cancer Epigenetics – Madrid (Spain)	Research	World wide	$>10^2$	IEO
23-25 March 2006	EMBO/SEMM Workshop on Homeodomain Proteins, Hematopoietic Development and Leukemias – Riva del Garda (Italy)	Research	World wide	$>10^2$	IEO
1-5 April 2006	AACR 97 th Annual Meeting 2006 – Washington, DC	Research	World wide	$>10^2$	IEO
2-5 April 2006	New Directions in Leukaemia Research – Brisbane (Australia)	Research	World wide	$>10^2$	IEO
26-26 May 2006	V Convegno Nazionale “Cellule Staminali e Progenitori Emopoietici Circolanti” – Roma (Italy)	Research	World wide	$>10^2$	IEO
13 June 2006	EHA IMC – Amsterdam (Netherlands)	Research	World wide	$>10^2$	IEO
22-24 June 2006	Breast Cancer and metastasis – Paris (France)	Research	World wide	$>10^2$	IEO
29 June 2006	SIES Discutiamone Insieme - Firenze (Italy)	Research	World wide	$>10^2$	IEO
4-5 September 2006	Targeted Therapies in cancer Myth or reality – Milan (Italy)	Research	World wide	$>10^2$	IEO
5 September 2006	First International Workshop on the Extension of Life Span Cesano Maderno (Italy)	Research	World wide	$>10^2$	IEO
20-23 September 2006	Second World Conference on the Future of Science – Venice (Italy)	Research	World wide	$>10^2$	IEO
29 Sept – 4 October 2006	14 th Euroconference on Apoptosis “Death or Survival?” – Chia (Italy)	Research	World wide	$>10^2$	IEO
5-6 October 2006	Epitron Internal Management Committee Strasburgo (France)	Research	World wide	$>10^2$	IEO
11-13 October 2006	6 th ISREC Conference on Cancer Research – Lausanne (CH)	Research	World wide	$>10^2$	IEO
1-4 November 2006	5 th Biennial International Sentinel Node Society – Rome (Italy)	Research	World wide	$>10^2$	IEO
4-8 November 2006	11 th International Conference on Differentiation Therapy and Innovative Therapeutics in Oncology – Versailles (France)	Research	World wide	$>10^2$	IEO
8-9 November 2006	Cancer Genomics Centre – Amsterdam (Holland)	Research	World wide	$>10^2$	IEO
15 November 2006	La Leucemia promielocitica quale modello per nuove	Research	World wide	$>10^2$	IEO

Third Annual Periodic Activity Report (2006)

	strategie antitumorali – Bellinzona (CH)				
17-18 November 2006	Liver Symposium Italiano – Rozzano (Italy)	Research	World wide	>10 ²	IEO
8-9 February 2005	Epigenetics and chromatin – Madrid (Spain)	Research	World wide	>10 ²	IEO
13-17 February 2005	Can cancer be treating as a chronic disease – Agra (India)	Research	World wide	>10 ²	IEO
4-5 February 2005	Keystone Symposia – Keystone – Colorado (USA)	Research	World wide	>10 ²	IEO
9-12 May 2005	VI MEETING OF MOLECULAR ONCOLOGY –Positano (Italy)	Research	World wide	>10 ²	IEO
5-7 July 2005	FEBS: Adaptor proteins and their functions – Budapest (Hungary)	Research	World wide	>10 ²	IEO
19-20 July 2005	BioScience2005 – Glasgow (Scotland)	Research	World wide	>10 ²	IEO
21-23 December 2005	1st WORLD CONFERENCE ON THE FUTURE OF SCIENCE – Venezia (Italy)	Research	World wide	>10 ²	IEO
28 September 2005	Meeting on Molecular Therapy – TRIESTE (Italy)	Research	World wide	>10 ²	IEO
3-5 October 2005	Mitochondria in Neurological Disease and Aging – NEW YORK (USA)	Research	World wide	>10 ²	IEO
7-8 November 2005	CNIO Cancer Conference – Madrid (Spain)	Research	World wide	>10 ²	IEO
28-30 April 2004	Conference on Diff. Therapy – Shanghai	Research	World wide	>10 ²	IEO
17-18 July 2004	From Fundamental Research To Cancer Therapy-Present&Future – Nizza	Research	World wide	>10 ²	IEO
8 September 2004	CNIO/Animal Model Call FP6 – Amsterdam	Research	World wide	>10 ²	IEO
18-19 September 2004	Metastasis Research Society Congress – Genova (Italy)	Research	World wide	>10 ²	IEO
4-5 October 2004	Cell-IMM Aging and Human Disease Meeting – Spineto (Italy)	Research	World wide	>10 ²	IEO
14 December 2004	Cancer Drug Discovery in Metabolic Pathways-Merck – Boston (USA)	Research	World wide	>10 ²	IEO

Section 3 – Publishable results

The integrated Project has so far not given rise to exploitable results.

Partner 13, Vichem (WP 32)

Section 1 – Exploitable knowledge at its use (month 36)

None of the results from the WP have lead to exploitable results or patents.

Section 2 -Dissemination of knowledge (month 36)

Publications (months 1-36)

There were no publications and presentations.

Section 3 – Publishable results

WP32 have so far not given rise to exploitable results.