EPITRON

EPIgenetic TReatment Of Neoplastic Disease

EPITRON Project

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Summary description of project objectives	The overall objective of EPITRON is to define and validate the concept of epigenetic treatment of cancer. To do this, the EPITRON consortium will: - *provide a molecular understanding of the genome-wide genetic and epigenetic consequences of the formation of onco-fusion proteins in AML* - *reveal the molecular mechanism of the anti-leukemic and anti-cancer action of epi-drugs* - *reveal the basis of the tumor-selective action of the TRAIL signaling pathway that is activated by epi-drugs, nuclear receptor ligands and interferons* - *establish mouse models to test the spectrum of anticancer actions of TRAIL in vivo and define in vivo toxicities* - *establish mouse models that faithfully report the activation of (cancer cell-selective) anti-tumor pathways* - *synthesize and validate novel epi-drugs* - *verify the concept of drug sensitization by signaling crosstalk* - *establish novel models to study epi-drugs in solid tumors* Thanks to the multi-disciplinary nature of the consortium, we are in a position to develop an entire pipeline to accomplish these goals—in addition to a strong grounding in basic research, our expertise includes drug design and synthesis, whole-genome approaches and high-throughput screening, clinical interface, and the development and use of mouse models which accurately reproduce the human disease.
Contractors involved	1 Centre Européen de Recherche en Biologie et Médecine - Groupement d'Intérêt Economique 2 Istituto Europeo di Oncologia Srl 4 Stichting Katholieke Universiteit 5 Universidade de Vigo 6 Seconda Università degli Studi di Napoli. Dipartimento di Patalogia generale 7 Centre Nationale pour la Recherche Scientifique 8 Università degli Studi di Milano 9 University of Cambridge 10 University of Turku 13 Congenia s.r.l. 14 Bayer Schering Pharma 16 Diagenode
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1. EPITRON PARTNERS – Work Performed and Results Achieved

In this section each partner summarizes the activities performed and contributions to the EPITRON project, the main publications resulting from this work are also indicated.

1.1 PARTNER 1 - CERBM-GIE:

Within the EPITRON consortium our laboratory has contributed to, and pushed forward common projects, in particular the development of an entirely novel triple action epi-drug. In addition we have (i) explored the mechanistic basis, epigenetic regulation, impact of aging and in vivo action of the tumor-selective apoptogenic action of the TRAIL signalling pathway that we had originally found to contribute to the action of a diverse set of cancer therapeutic compounds, such as retinoic acid action and HDAC inhibitors, (ii) developed novel therapeutic paradigms for cancer therapy, (iii) described a novel crosstalk between two epigenetic enzymes that results in diversification of the estrogen-regulated signalling pathway; future studies will reveal if this crosstalk constitutes a signalling code ("meHAT code"). Finally, faced by the present constraints of the ChIP-seq technology we (iv) have developed a novel technology that allows for the profiling of ultra-small compartments that are comprising a few hundred cells. Below some of the key issues are specifically addressed. Development and pre-clinical characterization of a novel triple action epigenetic inhibitor. Enzymes improperly depositing chromatin epigenetic marks during tumorigenesis are recognized therapeutic targets and the first 'epi-drugs' have entered the clinics. Together with three groups of EPITRON we have developed an entirely novel type of epi-drug that targets multiple epigenetic modulators simultaneously. The group of A. R de Lera has developed a single chemical entity (UVI5008) and in collaboration with L. Altucci's and H. Stunnenberg's groups we have shown that UVI5008 inhibits three distinct classes of epi-enzymes, histone deacetylases, sirtuins and DNA methyltransferases in vitro and in tumors in vivo. Importantly, UVI5008 displays cancer cell-selectivity inducing multiple tumoricidal pathways. This redundancy enables the triple inhibitor to target p53 and/or TRAIL wild-type and mutant/deficient tumor cells with similar efficacies. The activities of UVI5008 have been validated in vitro, in leukemia patients' blasts ex vivo, in human colon cancer xenografts and in primary tumors of breast cancer genetic mouse models. In keeping with its pharmacokinetics, the epigenetic activities of UVI5008 were demonstrated in tumors. Targeting multiple epigenetic enzymes by mono-therapy has promise as new therapeutic concept to target a wide range of tumor cells and to reduce the risk of drug resistance development. Clinical studies are in planning and contacts with pharmaceutical companies are ongoing [Gronemeyer et al., WO 2008/125988 A1]

Towards the meHAT code. Multiple signaling pathways ultimately modulate the epigenetic information embedded in the chromatin of gene promoters by recruiting epigenetic enzymes. We found that in estrogen-regulated gene programs, the acetyltransferase CREB Binding Protein (CBP) is specifically and exclusively methylated by the co-activator-associated arginine methyltransferase (CARM1) in vivo. CARM1-dependent CBP methylation and p160 coactivators were required for estrogen-induced recruitment to chromatin targets. Notably, methylation increased the histone acetyltransferase activity of CBP and stimulated its autoacetylation. Multi-dimensional comparative genome-wide ChIP-sequencing studies revealed a variety of patterns by which p160, CBP and methyl-CBP (meCBP) are recruited (or

not) by estrogen to chromatin targets. Moreover, significant target gene-specific variation in the recruitment of (i) the p160 RAC3 protein, (ii) the fraction of a given meCBP species within the total CBP and (iii) the relative recruitment of different meCBP species, suggest the existence of a target gene-specific "fingerprint" for co-regulator recruitment. Crossing of ChIP-sequencing and transcriptomics profiles revealed the existence of meCBP "hubs" within the network of estrogen-regulated genes. Together our data provide evidence for an unprecedented mechanism by which CARM1-dependent CBP methylation results in gene-selective association of estrogen-recruited meCBP species with different HAT activities and specifies distinct target gene hubs thus diversifying estrogen receptor programming. [Ceschin et al., Genes Dev 2011]

Autonomous rexinoid apoptosis. Growth factor (GF) deprivation and/or blocking of cognate signalling can induce apoptosis and is the basis of several cancer treatment paradigms. We observed that RXR agonists (rexinoids) induce apoptosis of tumor cells when GF support is abrogated. This "rexinoid apoptosis" involves activation of both iNOS and eNOS by RXR-PPARg and results in production of apoptogenic NO. IGF/EGF-induced IGF receptor 1-mediated MAP kinase blocks rexinoid apoptosis by RXR phosphorylation. Our results suggest a regulatory mechanism in which GF signalling antagonizes RXR-PPARg-mediated default apoptosis to sustain cell life. [Shakaranarayanan et al., Cancer Cell 2009 ; Gronemeyer, H. & Pattabhiraman, S. RXR-PPARgamma agonist/growth factor inhibitor combination therapy for inducing apoptosis and the treatment of cancer, Patent application 17.12.2008, Pub. No.: WO/2010/070379] Development of novel TRAIL-based paradigm for cancer therapy. Tumor-selective apoptosis is a major interest of my laboratory since our discovery of the convergence of multiple cancer therapeutic paradigms on the TRAIL signaling system. Our interest focuses on the following issues: (i) development of innovative TRAIL-based drugs, (ii) use of isogenic systems of normal and tumor cells to identify the factors required for TRAIL sensitivity of tumor cells, (iii) definition of a "TRAIL signature" that can be used for patient stratification, (iv) analysis of the TRAIL signaling system during the process of oncogenic transformation, and ultimately (v) understanding of the mechanisms accounting for the unique ability of TRAIL to selectively kill tumor cells.

Novel type of TRAIL-based tumor-selective apoptogenic drug. Tumor necrosis factor-Related Apoptosis Inducing Ligand (TRAIL/Apo2L/TNFSF10) engages its death-signalling receptors DR4 and DR5 to form trimeric complexes, thereby initiating the activation of the extrinsic apoptotic pathway. TRAIL displays the unique characteristic of inducing apoptosis in tumor cells while sparing normal ones. The cancer-therapeutic potential of the TRAIL pathway is currently explored in clinical trials with either recombinant human TRAIL, or humanized DR4/TRAIL-R1/TNFRSF10A or DR5/TRAIL-R2/TNFRSF10B-activating antibodies. We have used a different approach by developing small stable peptides that mimic the interaction surface of TRAIL with its death signalling receptors. As a result of this work we have found and reported a cancer cell-selective apoptogenic activity of multivalent DR5-selective peptides (TRAILmim/DR5) which was validated both in vitro and in vivo. Surface plasmon resonance assays revealed up to several thousand-fold increased affinities of two TRAILmim/DR5-receptor complexes upon generation of di and trivalent molecules, the latter of which was done by using a conformationally-restricted adamantane core. Notably, only divalent and trivalent molecules triggered a substantial DR5-dependent apoptotic response in vitro. By

using stepwise tumorigenesis models derived from human embryonic kidney cells and human foreskin fibroblasts, we demonstrated the tumor-selective action of TRAILmim/DR5 peptides and their ability to synergize with resveratrol. We showed that the tumor-specificity of the peptides is retained upon resveratrol sensitization, the latter of which takes place in a p53 integrity-independent manner. Finally, validation of the in vivo anti-cancer activity of a divalent TRAILmim/DR5 with xenografted human colon cancer cells revealed a major growth inhibition. Our results represent a proof-of-principle for the potential use of small synthetic molecules to trigger the tumor-selective TRAIL pathway for cancer therapy. [Pavet et al., Cancer Res 2010]

Transformation-dependent epigenetic silencing of TRAIL. Albeit the TNF-related apoptosis-inducing ligand TRAIL (TNFSF10) kills malignant cells selectively, tumor cells can escape this control. Using multiple stepwise human cellular tumorigenesis models we asked which mechanisms are responsible for this escape from apoptosis. In the course of this study we observed DNA methylation-dependent silencing of TNFSF10 concomitant with HRASG12V–mediated transformation, indicating that emerging tumor cells may escape elimination by epigenetic inactivation of the TRAIL pathway. Indeed, in silico comparison of leukemia, lung and colorectal cancers with the normal tissues revealed widespread down-regulation of TNFSF10 expression, while DNA demethylation or DNMT ablation reactivated expression of TNFSF10 and pro-apoptotic regulators of the TNFSF10 pathway. In vivo treatment of xenografted HRASG12V-transformed human epithelial kidney cells, primary and metastasized human colon carcinomas or syngenic mouse tumors by decitabine blocked tumor growth, and induced both TRAIL expression and apoptosis. These results provide a rationale for combination therapies with decitabine to increase tumor-selective apoptosis. [Lund et al., 2011 - Mol Cancer Ther, Jun 22.]

LinDA for the ChIP sequencing of ultra-small samples. Presently, ChIP-sequencing requires nanogram quantities of DNA. In the context of EPITRON we want to profile transcription factors and epigenetic modifications in analysis of very small cell compartments. For this we developed and validated LinDA, a novel versatile single-tube linear DNA amplification method, for ChIP-seq, re-ChIP-seq and related technologies with picogram DNA amounts. This amplification technology will facilitate global TF binding and chromatin analyses with very limited cell populations, such as stem or cancer initiating cells. [Shankaranarayanan et al., Nature Methods 2011]

List of selected publications:

Ceschin DG, Walia M, Wenk SS, Duboé C, Gaudon C, Xiao Y, Fauquier L, Sankar M, Vandel L, Gronemeyer H. Methylation specifies distinct estrogen-induced binding site repertoires of CBP to chromatin. Genes Dev. 2011 Jun 1;25(11):1132-46.

Lund P, Kotova I, Kedinger V, Khanwalkar H, Voltz E, Hahn WC, Gronemeyer H. Transformation-dependent Silencing of Tumor-selective Apoptosis-inducing TRAIL by DNA Hypermethylation is Antagonized by Decitabine. Mol Cancer Ther. 2011 Jun 22.

Pavet V, Beyrath J, Pardin C, Morizot A, Lechner MC, Briand JP, Wendland M, Maison W, Fournel S, Micheau O, Guichard G, Gronemeyer H. Multivalent DR5 peptides activate the TRAIL death pathway and exert tumoricidal activity. Cancer Res. 2010 Feb 1;70(3):1101-10. Epub 2010 Jan 26.

Shankaranarayanan P, Mendoza-Parra MA, Walia M, Wang L, Li N, Trindade LM, Gronemeyer H. Single-tube linear DNA amplification (LinDA) for robust ChIP-seq. Nat Methods. 2011 Jun 5;8(7):565-7.

Shankaranarayanan P, Rossin A, Khanwalkar H, Alvarez S, Alvarez R, Jacobson A, Nebbioso A, de Lera AR, Altucci L, Gronemeyer H. Growth factor-antagonized rexinoid apoptosis involves permissive PPARgamma/RXR heterodimers to activate the intrinsic death pathway by NO. Cancer Cell. 2009 Sep 8;16(3):220-31.

Patent WO 2008/125988 A1, international publication date 23.10.2008: Novel derivatives of Psammaplin A, a method for their synthesis and their use for the prevention or treatment of cancer.

1.2 PARTNER 2 – EIO1:

Summary of the contribution from Partner 2.1(SM) during the entire duration of the project. Partner 2.1(SM) has largely collaborated with several partners and achieved all the milestones associates with his WPs and tasks. Indeed, many of the studies performed have already been published or have reached a publication stage level.

WP1: within this WP we extensively collaborated with partner 2.2 (PGP), in particular for tasks 2 (Chip-Seq analysis of oncofusion proteins) and 3 (Comparative global methylome analyses).

WP2: We have set up a novel technology (NA-Seq) for the study of genome-wide chromatin accessibility to exogenous nucleases (Gargiulo G. et al., 2009). This technique offers a synthetic view of chromatin organization, complementing the more detailed view that can be achieved only by the systematic analysis of histone marks. We have further improved and adapted the technology to the Illumina-Solexa platform for a higher throughput and complete genome saturation: currently, we have also miniaturize this approach (and conventional ChIP techniques), for the study of small cell populations (patient samples, rare cancer stem cells). The results described above have been the basis for studies aimed to annotate novel markers for functional DNA elements. Indeed, we identified H2AK5Ac (manuscript in preparation) as a histone PTMs found at functional elements independently from their genomic position (Fig. 1), and we demonstrate that H2AK5ac distribution well correlates with active gene expression, in a cell-type and stage-specific manner (Fig. 2). Rcently, we have developed a new ChIP procedure (PAT-ChIP) where chromatin is prepared from paraffin-embedded tissue samples (Fanelli M. et al., PNAS, 2010). We chose these samples because they represent a very important source of material on which it is possible to perform studies aimed to discovery new potential biomarkers. Indeed, archives of paraffin-embedded tissues are present in every hospital, being to date the best method to preserve material for histological analysis. Unfortunately, this technique is not considered suitable for preserving delicate cellular characteristics such as chromatin structure. However, we succeed in developing a protocol for the isolation of chromatin from paraffin-embedded tissues with purity compatible to perform fine epigenetic studies such as ChIP-Seq. This technique will be very useful for epigenetic studies performed on patient's tissues allowing clarifying the role played by the different epigenetic alterations during the development of the disease. At present, we have validated and applied this technology at both single gene and genome-wide (ChIP-Seq) levels.

WP4: in a preclinical mouse model of acute myeloid leukemias (APL), we have shown that: a) monotherapies of either HDACi (VPA) or DNMTi (DAC) induced increased survival in APL mice and their combination was able to further prolong survival of APL mice, demonstrating the importance of hitting multiple targets of the epigenetic "Silencing Loop"; b) at molecular level both VPA and DAC induced an apoptotic response in leukemic cells through the up-regulation of members of the Death Receptor pathways, in particular TRAIL. In the last year we focused our attention in studying whether distinct subpopulations within the leukemic mass show differential sensitivity to epigenetic drugs (in collaboration with HdT). To this purpose, we set up an in vivo leukemia transplantation mouse model. We used leukemic

cells derived from mGC-PML/RARA mice which are Ly5.2, and transplanted them in the congenic strain C57BL/6J-Ly5.1. In this way it is possible, by facs analysis, to detect donor-derived APL blasts. This information allows us to determine in the best way when to start the epigenetic treatment. After epigenetic treatment the purified leukemic blasts (expressing the common leucocyte antigen Ly5.2) are intravenously inoculated into secondary recipients (Ly5.1+) at different dilutions in order to establish the effect of the in vivo administration of the drug on the LICs through their transplantation capacity (Figure 4). Specifically, we studied two drugs recently approved by FDA for treatment of cancer patients, valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA). The different HDAC inhibitors showed a very different biological outcome as demonstrated by the free-survival curves showed in figures 5 and 6 (manuscript in preparation). Indeed, VPA, a HDAC class I inhibitor, does not induce LIC clearance. However, ongoing experiments suggest that if administered in combination with All Trans Retinoic Acid (ATRA), it synergizes in LIC eradication. On the contrary, SAHA, a compound with specificity for both class I and II HDACs, can trigger LIC loss, as demonstrated by its ability in diminishing their capability to propagate the disease following transplantation. To further investigate the interplay between PML-RAR and HDACs, and in particular to understand the different contribution to APL development played by the different HDACs from class I, we have performed a selective knock down of HDAC1, 2 and 3 through RNA interference in Lin- cells derived from mouse expressing the oncogene fusion protein PML-RARa (P/R KI mice) (manuscript in preparation). Surprisingly, in this study we demonstrated that in the pre-leukemic stage, HDAC1 is required to counter attack PML-RAR oncogenic effect as shown by the acceleration of APL development in mice reconstituted with lin- cells expressing PML-RAR and knocked down for HDAC1. Moreover, as soon as 30 days post transplantation of lin- cells expressing PML-RAR and knocked down for HDAC1 these cells acquire an immunophenotype close to APL blast (double positivity for Gr-1 and c-Kit surface markers) together with a reduced ability to differentiate along the myeloid lineage, and increased proliferative potential (Fig.7a,b). These results, suggesting that HDAC1 behaves as a tumor suppressor, have important implication in the development of HDAC isoforms specific inhibitors. All together these data demonstrate that different HDACs may participate in different ways to APL development.

WP5: during the project, we have extensively collaborated with different groups in the follow way: i) we have studied (in collaboration with A. Costanzo (Uni Roma) and Congenia) a basal cell carcinoma derived murine cell line, that we have characterized in terms of expression of several epigenetic modifiers, and of response to epigenetic drugs; ii) melanoma cell lines have been derived from patients and have been used to test epigenetic drugs. These results have been described in another section of the report (Congenia); iii) we have establish a murine model systems for the study of breast cancer stem cells (described by partner PGP). This model is at the basis for the study of the effect of epigenetic drugs on breast cancer stem cells. Regarding to our contribution for the studies of human breast cancer we have: i) set up a quantitative assays to measure the response to HDACi of primary breast cancer cultures; ii) analyzed the sensitivity of bulk primary breast cancer cultures to HDACi and correlated it to their epigenetic status. Main results: breast cancer patients show different responses to HDAC inhibitors. While some are sensible to treatment, others are totally resistant. Our studies showed an association between chromatin acetylation levels and treatment response (fig 8). Starting from gene expression profiles from 100 patients

divided into acetylated and hypoacetylated we performed a bioinformatics analysis which allow us to identify a small combinations of genes for the acetylated class as well as for the hypoacelytaed class that are able to predict sensitivity to the drug. Recently, we concluded an in vitro study aimed to explain the mechanism of action of HDACi. In this work, performed in mammal cells, we showed that systemic administration of histone deacetylase inhibitor causes heterochromatin relaxation, increased DDR activation, apoptosis and tumor regression. We propose a model (fig 9) in which oncogenic stress-induced heterochromatin restrains DDR and this may provide the rationale for the use of chromatin modifying drugs, including HDACi, as cancer therapies (Di Micco R. et al.).

WP6: During this period we have collaborated with other partners for the achievement of the proposed goals. In particular we contributed to the setting-up for both crystallization procedures and enzymatic activity assays. We also supplied initial amount of protein and construct to perform preliminary studies. Please refer to Congenia's report for more details.

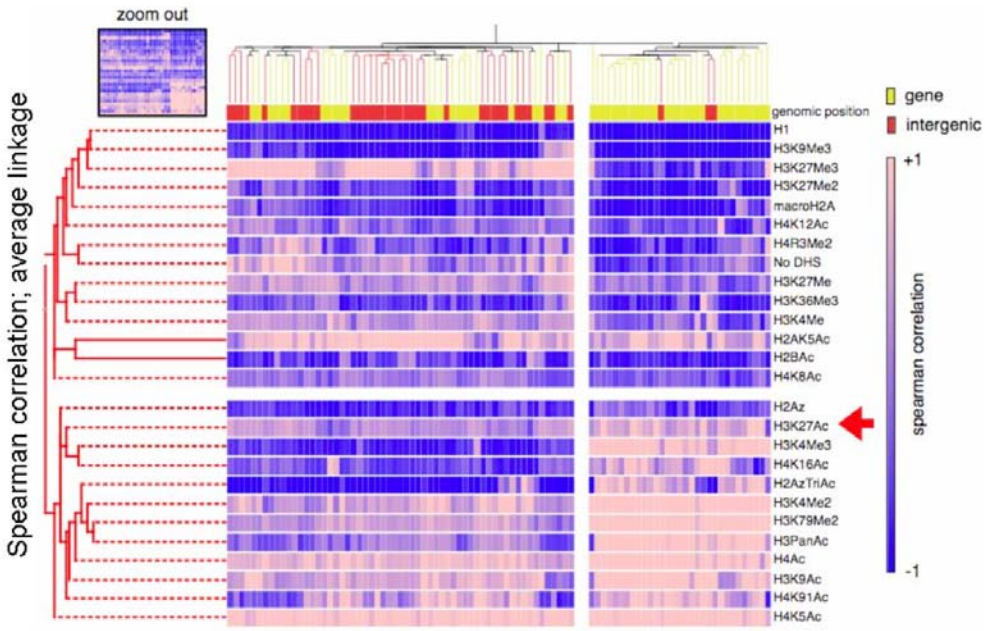


Fig.1 NAS are acetylated at H2AK5 (independently from their genomic position).
Spearman correlation; average linkage

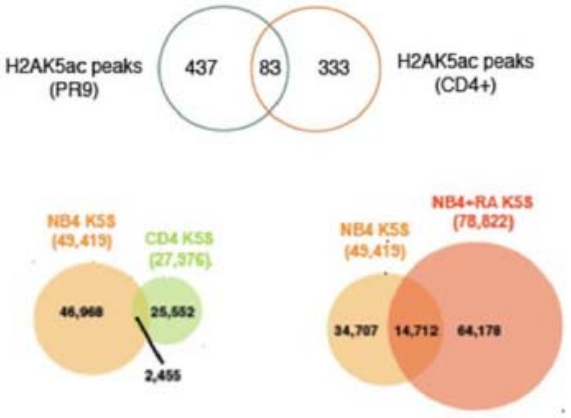


Fig. 2 Distribution of K5S changes according to cell-type and stage. K5S associates with active gene expression (profiling studies)

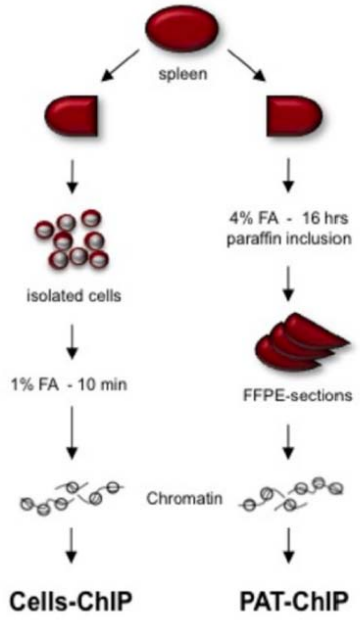


Fig 3. Schematic representation of canonical ChIP and PAT-ChIP procedures, starting from spleens of leukemic mice.

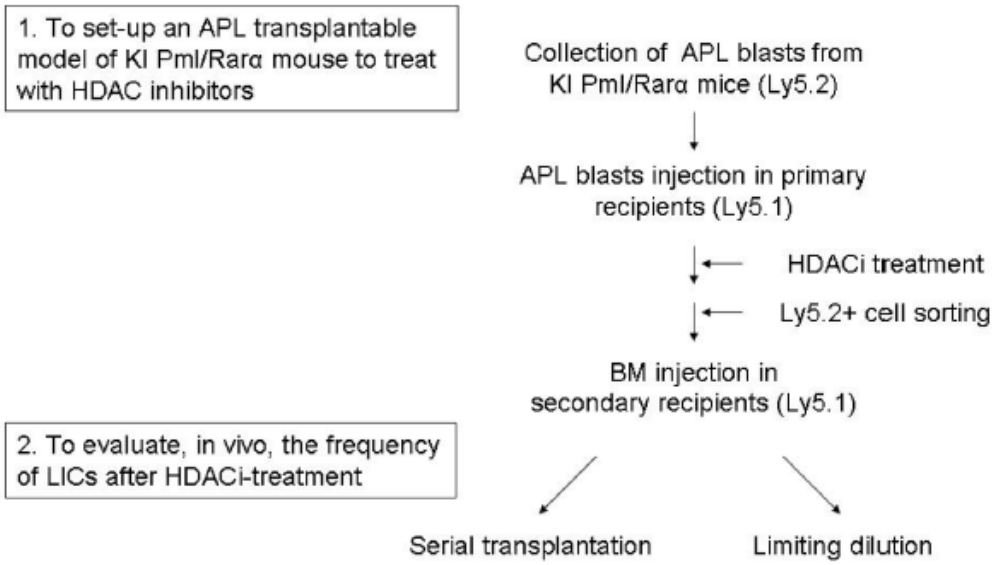


Fig4. APL transplantation murine model for the sensitivity study of different leukemic sub populations

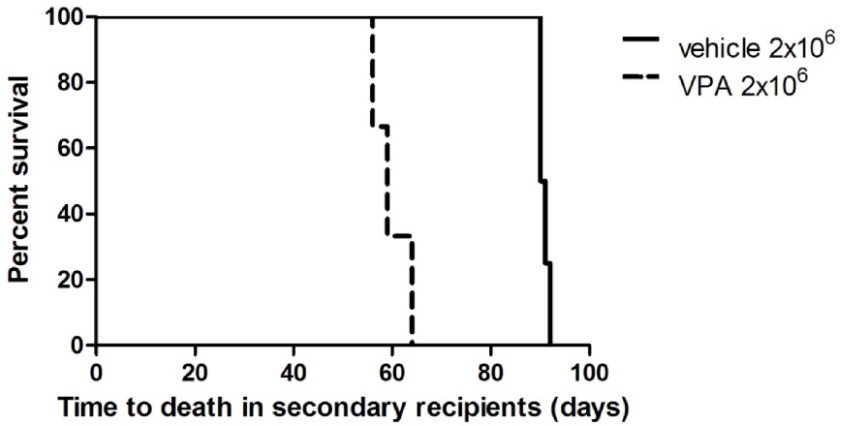


Figure 5 Leukema free survival curves of VPA treated leukemic mice

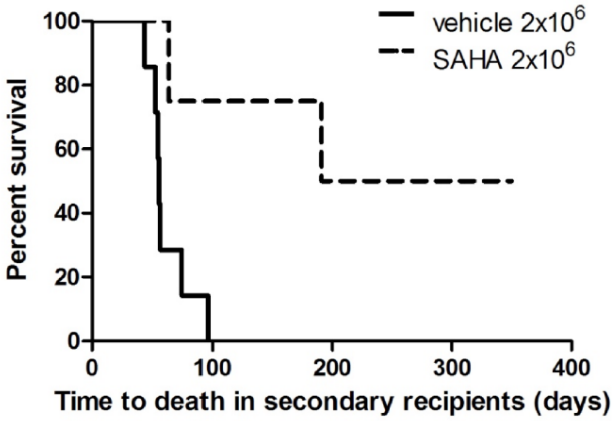


Figure 6 Leukema free survival curves of SAHA treated leukemic mice

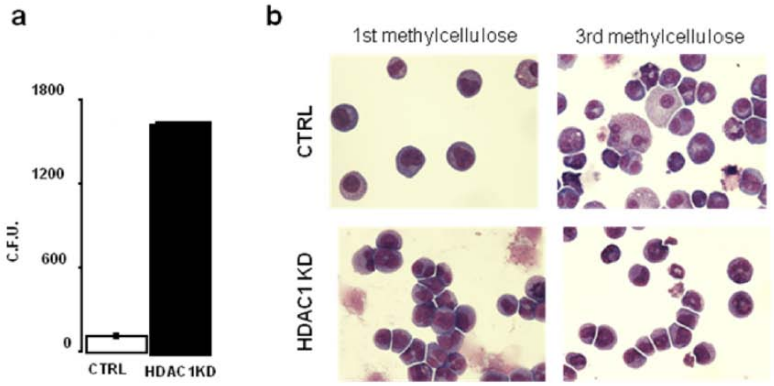


Figure 7

30 days after transplantation Gr-1 and c-kit double positive cells were sorted and analysed for their proliferative capability (a) and differentiation properties (b).

Figure 8 Association between chromatin acetylation levels and treatment response. Upper panel Breast cancer classification based on global histone acetylation . Lower panel HDAC sensitivity correlates with global levels of chromatin acetylation (R=resistant; S=sensitive)

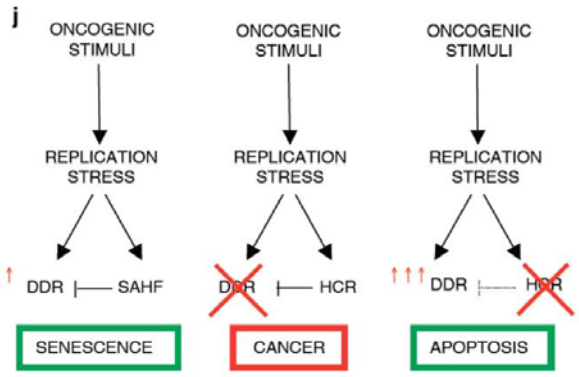


Figure 9

Proposed model of the events

following oncogene activation. Oncogene activation through DNA-replication stress leads to OIS associated with DDR activation, global induction of heterochromatin (HCR) and SAHF formation. SAHF restrain DDR activity. DDR inactivation allows proliferation and transformation of oncogene-expressing cells maintaining heterochromatin induction. Heterochromatin perturbation, relieving heterochromatin inhibitory effect on DDR, increases DDR signaling and leads to apoptosis.

List of selected publications:

Gargiulo G, Levy S, Bucci G, Romanenghi M, Fornasari L, Beeson KY, Goldberg SM, Cesaroni M, Ballarini M, Santoro F, Bezman N, Frigè G, Gregory PD, Holmes MC, Strausberg RL, Pelicci PG, Urnov FD, Minucci S. NA-Seq: a discovery tool for the analysis of chromatin structure and dynamics during differentiation. Dev Cell. 2009 Mar;16(3):466-81.

Fanelli M, Amatori S, Barozzi I, Soncini M, Dal Zuffo R, Bucci G, Capra M, Quarto M, Dellino GI, Mercurio C, Alcalay M, Viale G, Pelicci PG, Minucci S. Pathology tissue-chromatin immunoprecipitation, coupled with high-throughput sequencing, allows the epigenetic profiling of patient samples. Proc Natl Acad Sci U S A. 2010 Dec 14;107(50):21535-40. Epub 2010 Nov 24.

Di Micco R, Sulli G, Dobreva M, Liontos M, Botrugno OA, Gargiulo G, dal Zuffo R, Matti V, d'Ario G, Montani E, Mercurio C, Hahn WC, Gorgoulis V, Minucci S, d'Adda di Fagagna F. Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. Nat Cell Biol. 2011 Mar;13(3):292-302. Epub 2011 Feb 20.

1.3 PARTNER 2.2 - EIO2:

During the period of activity, we have been mainly involved in Workpackages (WP) 1, 2, 5 and 11. The objective of our research within WP1 was to apply ChIP-based technologies to look into the target gene programme of two leukemia-associated fusion proteins, AML1-ETO and PML-RAR. For the analysis of AML1/ETO, we used a U937 cell line that conditionally expressed HA-tagged AML1/ETO under the control of a Zn-inducible promoter (U937-A1E). At first, we performed whole genome ChIP-on-chip experiments, using an anti-HA antibody and the NimbleGen Human Promoter Two-Array set, and then we used Affymetrix GeneChip U133 2.0 to evaluate genes differentially expressed in U937-A1E cells compared to control cells, which carried an inducible empty vector. Through the integration of ChIP-on-chip and expression profiling results, we have identified 358 putative direct target genes, of which 247 downregulated and 111 upregulated by AML1/ETO through unknown mechanisms. A detailed sequence analysis has revealed a specific sequence signature associated with genomic occupancy of AML1/ETO, which included a significant enrichment for AML1 and HEB binding sites. Interestingly, AML1/ETO expression results in displacement of HEB and AML1 from these common regions at lower frequency than in genomic locations where these transcription factors do not co-localize. We thus examined the correlation between gene expression and DNA binding pattern of AML1/ETO, AML1 and HEB by expression tiling. We have showed that the expression level of HEB target genes could be modified by AML1/ETO expression, suggesting that transcriptional regulation determined by AML1/ETO may partly derive from displacement of HEB from its native binding sites. These results pointed out at a crucial role for AML1 and HEB in AML1/ETO-dependent transcriptional regulation underlying myeloid leukemogenesis. For the target gene programme of PML-RAR, we began using a gene ChIP technology (chromosome 19 tiling array). We found 726 genes regulated by the fusion protein, the majority lying in adjacent genomic regions, suggesting that PML-RAR is able to cause long-range chromatin alterations. Through a computational analysis, we showed that PML-RAR binding sites were flanked by RARE (Retinoic Acid Response Element) clusters that coincided with Alu clusters, and were able to strongly bind the fusion protein. This resulted in changes in higher order chromatin structure that induced proximity among distant genomic regions through the formation of multiple DNA loops. By a ChIP-seq analysis on control and PML-RAR-expressing PR9-cells, we have identified 3,558 direct targets of the leukemogenic fusion protein. Through the integration of the PML-RAR binding sites with the dataset of regulated PML-RAR target genes (previously obtained by an Affymetrix screening) we have characterized 64 large genomic “domains” identified by enriched regions of genes down-regulated and PML-RAR bound and surrounded by cluster of AluRARE. We have demonstrated that PML-RAR binding occurs, preferentially, in the proximity of the transcription start site (TSS) of those genes. These results have been confirmed on leukemic cells (NB4) derived from an APL patient by ChIP-seq experiments. In fact, we have demonstrated that the same cluster-associated chromatin changes found in the model cells, few hours after PML-RAR induction were maintained in fully transformed leukemic blasts. To further characterize the molecular features of these interactions, we performed an unbiased motif search analysis under the regions bound by PML-RAR. Our results showed an enrichment of SPI1/PU.1 responsive elements. Importantly, an extensive analysis of these data has shown that PML-RAR and SPI1/PU.1 share at least 30% of the binding regions of PML-RAR. Since SPI1/PU.1 appears specifically bound to 50% of PML-RAR

direct targets genes, we suggest that it drives the binding of PML-RAR to promoters where RARE elements are not present. We thus propose a working model in which PML-RAR at first binds to a specific sequence of the AluRARE clusters and then, possibly by an anchorage mechanism driven by SPI1/PU.1, which is already on the promoter of PML-RAR target genes, induces chromatin looping on the same promoters. Main objective of our research activity in WP2 (closely linked to WP1) was to provide a mechanistical analysis of chromatin dynamics and long range/global modifications in the chromatin structure caused by PML-RAR. We have confirmed by chromosome conformation-capture and 3D FISH assays that long-range interactions take place between Alu-RARE clusters and target genes hundreds of Kb away along the DNA fiber and we have demonstrated that these alterations could be reverted, or at least significantly reduced, by retinoic acid treatment. Since these long-range interactions, by generating persisting DNA loops, might constitute an obstacle for DNA replication and might contribute to DNA damage, we have monitored the time-dependent accumulation of gamma-H2AX, a specific histone mark of DNA damage, in an asynchronous cell population. We have found a striking co-localization between the position of the genomic sites containing gamma-H2AX: and i) our genome-wide PML-RAR binding profile; ii) clusters of genes down-regulated by PML-RAR that we had previously identified; iii) AluRARE clusters. Since this general reorganization of the chromatin, following PML/RAR expression, might have an impact not only on global transcription patterns, but also on global repositioning of nuclear domains that takes place before DNA replication, we have also focused our attention on the analysis of the effects of oncogenic factors on the replication of cancer cells. Therefore, we have started an ambitious study of human replication origins (ORIs), beginning with the development of a new method to isolate ORIs, based on chromatin fractionation in a density gradient. We focused on Orc1, a protein that regulates origin recognition complex (ORC) activity in a cell cycle dependent manner and select the origins that will “fire” in the following S phase. We have identified 1,850 bona fide Orc1-binding sites in human U937 cells. Validation rate of Orc1 positive sites was higher than 95%, and analysis of origin activity with an independent assay (Nascent Strand Abundance assay) has shown that almost all of them were active replication origins. Interestingly, 52% of the Orc1 peaks identified by ChIP-seq analysis map within gene promoters. We have used our recently developed methodology to identify ORIs in the presence or absence of PML-RAR expression, in order to check if PML-RAR modifies normal replication pattern of cells by affecting the mechanism of origin selection. By a comparative analysis we have found that more than 50% of ORIs that had been mapped in control cells were lost upon PML-RAR expression, while thousands of de novo-firing ORIs were identified. To verify if PML-RAR is able to alter the normal replication timing of the human genome, globally and/or within “repression domains”, we have performed a RepliSeq assay (which provides high-resolution DNA replication patterns with respect to both cell-cycle time and genomic position) in the presence or absence of PML-RAR expression in PR9 cells. We have also actively contributed to WP5. The specific goal of our research was to investigate how somatic stem cells and progenitors respond to DNA damage (DD) and oncogene expression or, in other words, how (and if) a DD- and oncogene-induced checkpoint is executed in these cells. We hoped to elucidate some of the earliest events of the carcinogenesis process. Our studies were prompted by our recent demonstration that expression of leukaemia-associated oncogenes induces DNA damage in hematopoietic stem cells, without, however, leading to exclusion of damaged cells, due to a (p53-independent) activation of the cell-cycle inhibitor p21 and to

repair of damaged DNA (Viale et al., Nature 2009). Since this property of p21 is specific to stem cells, these data suggested a unique regulation of DNA damage-dependent checkpoints in stem cells. We set to test this hypothesis in two model systems which have been extensively characterised in our group: the mammary gland (breast stem cells, BSCs) and hematopoietic tissue (hematopoietic stem cells, HSCs). Specifically, we have investigated checkpoints activation in stem cells vs. progenitors after DNA damage (X-ray treatment) or oncogene expression (overexpressed Myc and ErbB2). We have shown that DNA damage induction by in vivo X-ray treatment of mice, activated different response mechanisms in Long Term reconstituting HSCs (LT-HSCs) with respect to progenitor cells (MPPs and CMPs). In fact, in LT-HSCs we observed an upregulation of the cell cycle inhibitor p21 without induction of p53, whereas, in progenitors p21 activation was p53 dependent. Furthermore, despite the presence of DNA damage (revealed by means of gamma-H2AX immunostaining), irradiated LT-HSCs did not undergo massive apoptosis as the progenitors; this resistance seemed to be due to a p21-dependent and p53-independent mechanism, as showed by experiments on p53-/- and p21-/- mice. We have thus developed and employed a high content image cytometry approach to evaluate the correlation between DNA damage induction (gamma-H2AX quantification) and response (detection of p53 activation through phosphorylation) and to investigate the potential protective effect induced by cell quiescence through the quantification of DD response in actively proliferating cells. Interestingly, we have been able to demonstrate that resistance to apoptosis was not due to less DD in stem cells after X-rays compared to more differentiated cells, or to quiescence of LT-HSCs. Notably, we have shown that protection from apoptosis in these cells is non-transitory and p21-dependent. We have confirmed our data by an analysis of damaged BSCs by in vitro experiments with mammosphere cultures. We found that the X-ray treatment induced a massive apoptosis in most cells (progenitors) but did not affect the capacity of BSCs to reform mammospheres and, as for the HSCs, this survival was p21 dependent and p53 independent. Moreover p21 appears to be important also for the modulation of repair processes in both HSCs and BSCs. Collectively, our results suggest that p21 is critical for apoptosis resistance, functional competence and DNA repair response of irradiated SCs (manuscript in preparation). Regarding oncogene expression, we have examined the effect of c-Myc overexpression in mammary epithelial cells (mammosphere cultures), both using a tamoxifen (4-OHT) inducible lentiviral system and a constitutive system, where cells have been infected with a constitutive construct for Myc expression under a CMV promoter, using different amounts of viral supernatant to achieve different levels of Myc expression. Unexpectedly, we observed that MycER-cells, expressing low levels of Myc showed a marked increase in their self-renewing and replicative potential (symmetric mode of division). On the contrary, cells that expressed high levels of Myc, showed an apoptotic response, associated with up-regulation of the p16 and p19Arf-p53 checkpoint pathways. Importantly, we have found that the expression of low levels of Myc in progenitor cells conferred on them stem-like properties, as demonstrated by their ability to form mammospheres and to repopulate mammary gland in vivo. Recently we have shown that normal mammary SCs rapidly lost self-renewal potential in culture, while cancer SCs are nearly immortal and their number expand geometrically, due to increased frequency of symmetric divisions. We have shown that the tumour suppressor p53 controls the modality of division in stem cells and that pharmacological restoration of p53 in cancer SCs restores the normal kinetics of self-renewal, without significantly affecting the growth of progenitor cells. Importantly, this

correlated with reduced tumour growth in vivo. These data indicated that: (1) extended life span and increased frequency of symmetric self-renewing divisions in cancer SCs are critical for tumour growth; (2) asymmetric division functions as a mechanism of tumour suppression in mammals (Cicalese et al., Cell 2009). We have thus investigated p53 involvement in the response to Myc by expressing low or high levels of Myc in p53 knockout mammospheres. Our results show that low levels of Myc expression have no additive effect on already immortalized p53 knockout mammospheres, suggesting that the pathway of immortalization may proceed through similar routes. Furthermore, and as expected, tamoxifen inducible expression of MycER in p53 knockout mammospheres did not trigger a pro-apoptotic checkpoint, suggesting that p53 is entirely responsible for the activation of this response in normal mammospheres. We had also planned to investigate the effect of epigenetic drugs on breast stem cells. We have analyzed the effect of two HDAC inhibitors, valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA), on WT mammary SCs, using the mammosphere system. High doses of both these inhibitors on mammosphere cell suspensions drastically reduced both the number and the dimension of neo-formed spheres, thus suggesting a toxic effect on SC and progenitor cells. Replating of the treated spheres without the drugs demonstrated that there are no further effects on the modality of SC divisions. In contrast, low dose treatments did not have any effect on WT SCs or progenitor cells. In order to repeat the treatment with the epigenetic drugs on cancer SCs, we are currently characterizing two new mouse models with respect to i) their ability to form mammospheres, ii) SC frequency in mammary tumors and mammospheres, and iii) SC PKH segregation. Finally, it is very important to underline that these major results would not have been achieved without the contribution of our bioinformatic unit (WP11). In particular, extensive bioinformatics analysis has been performed to support the activities of WP1 and WP2 and has enabled us to extract and analyze the massive amount of data derived from ChIP-based approaches and novel high-throughput sequencing technologies. Bioinformatics tools have proven to be crucial for the ChIP-on-chip analysis of oncogene binding sites and for the integration of these data with gene expression information. We also developed the PeakPicker software as a tool to perform peak analysis on different types of ChIP-on-chip platforms. During the period of activity, we have decided to replace the ChIp-on-chip technology with ChIP-seq, a technology based on chromatin immunoprecipitation combined with high-throughput sequencing. This approach was considered more efficient than ChIP-on-chip, as ChIP-seq results are characterized by a high signal/background ratio that renders the peaks easy to score. The intense efforts of our bioinformaticians have allowed us to develop new protocols and employ new technology platforms in a relatively short time. In conclusion, we have overall achieved the objectives that were set by exploring the mechanisms underlying the action of leukaemia-associated oncogenes, characterizing stem cell unique checkpoints and setting up new high-throughput technologies. These important results are opening the way to new researches. Moreover, in the course of the EPITRON project we have also consolidated important and productive collaborations with the other European partners.

List of selected publications:

Viale A, De Franco F, Orleth A, Cambiaghi V, Giuliani V, Bossi D, Ronchini C, Ronzoni S, Muradore I, Monestiroli S, Gobbi A, Alcalay M, Minucci S, Pelicci PG. Cell-cycle restriction limits DNA damage and maintains self-renewal of leukaemia stem cells. Nature. 2009 Jan 1;457(7225):51-6.

Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, Brisken C, Minucci S, Di Fiore PP, Pelicci PG. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell. 2009 Sep 18;138(6):1083-95.

1.4 PARTNER 4 - RU:

Objectives: - To establish the ChIP-seq technology with NB4 cells (model for APL) - To generate ChIP-seq profile for the target sites of PML-RARa and AML-ETO (using human blasts with the corresponding translocations) - To compare the target gene programs of PML-RARa, (PML) and the retinoic acid receptor alpha (RARa) by using ChIP-seq - To compare the target gene programs of AML-ETO, AML1 and ETO - To identify novel candidate drug targets with the ChIP-seq approach. - To perform comparative global methylome analyses for patients with patient blasts vs normal CD34+ cells. Establish the ChIP-chip and ChIP-seq technology. The original scope of this task was to establish the ChIP-chip technology. However, due to new technological developments this deliverable has been changed into establishing the ChIP-seq technology. As a result both ChIP-chip and ChIP-seq have been successfully established within the Epitron project. The establishment of both techniques has led to several publications (see below), amongst which Hatzis et al., 2008; Smeenk et al., 2008 (ChIP-chip); Welboren et al., 2009; Martens et al., 2010; Martens et al., submitted; Saeed et al., submitted (ChIP-seq). ChIP-chip initially, the main technique to examine genome-wide protein binding was based on combining chromatin immunoprecipitation (ChIP) with DNA hybridization to tiled oligonucleotide microarrays (ChIP–chip). Although this technique allowed global analysis of chromatin-associated proteins including transcription factors and modified histones, the spatial resolution and genomic coverage per microarray was relatively low. Moreover, as it requires multiple arrays to cover a complete genome of higher eukaryotes, the technique is very costly in order to generate comprehensive views on protein activity on chromatin. Still, ChIP-chip technology has been successfully established in the lab of partner 4, and complete data sets were generated and validated for the Estrogen Receptor. Initially, custom microarrays were generated, while at a later stage the hybridization facility of NimbleGen was utilized. Protocols for amplification were optimized and implemented. In particular the switch from LM-PCR based (exponential amplification) to T7-based linear amplification methods has proven to result in lower background without significantly altering the signal over noise ratio. The improved technology has been made available to other labs in the consortium as part of collaborative actions. ChIP-seq Despite the successful establishment of the ChIP-chip technique, a new and better technique for global binding site mapping, called ChIP-seq, emerged during the Epitron project. Partner 4 established this ChIP-sequencing technique using Solexa methodology in his laboratory. ChIP-seq offered several advantages over ChIP-chip. i) Solexa sequences directly the ChIPed material, whereas for ChIP-chip you first need to amplify your material (and possibly introducing a bias) and perform hybridizations (another potential bias). ii) For Solexa you can sequence everything, for ChIP-chip you can only examine whatever is on your arrays (for example: repeats will be sequenced in Solexa but cannot be found on a whole genome chip set from NimbleGen) iii) ChIP-chip material was send to NimbleGen/Iceland and we were depending on them, Solexa sequencing is done in house, so we have a better control over the experiment. iv) ChIP-chip takes about 4 weeks between sending of samples and receiving results. Solexa can be done within a week. v) Results from the Solexa look better as there is no background: whatever is sequenced was in your sample. For ChIP-chip you can also get crosshybridization to probes that are not represented in your ChIP sample but are present on your array. Indeed comparisons made between genome-wide ChIP-chip experiment, ChIP-Solexa done in the lab of partner 4 and ChIP-Solexa done by Illumina revealed superior

results for the ChIP-seq approach. Antibodies for ChIP-chip and ChIP-seq A crucial step in the ChIP procedure is the selection of the antibodies. Within Epitron different antibodies were developed by Epitron partners as well as companies such as Abcam and Diagenode. In the initial phase of Epitron (year 1-2) the goal was to find antibodies of ChIP-chip quality for PML-RARa and AML1-ETO. PML-RARa For PML-RARa we tested in ChIP different antibodies for PML, such as PGM3 and H238 from Santa Cruz, 298 from partner Hugues de The and A136 and A137 from partner Diagenode. Our initial analysis showed that the available PML and RARa antibodies where not ChIP-chip grade as the ratio signal/background in ChIP assays was not sufficient, i.e. lower than 5 fold. Still some of these antibodies worked well in ChIP in situations where the PML-RAR protein was overexpressed. As we aimed to examine genome-wide binding of PML-RAR in NB4 cells (which reflects more the situation in patients) the low level PML-RAR expression in these cells requires a very strong antibody before one can go into genome-wide studies, and therefore omitted the ones that we had analyzed. Together with the new Epitron partner Diagenode we designed and generated ~10 more antibodies against PML-RAR for testing in ChIP, ChIP-chip and ChIP-seq. Moreover, partner HdT provided us with newly developed antibodies for this purpose. Ultimately, this led to the identification of one ChIP-seq grade antibody (see below) for PML (H238 from Santa Cruz) and one ChIP-seq grade antibody (see below) for RARa (A704 from Diagenode). AML1-ETO. For AML1-ETO we planned to use SKNO-1 and Kasumi-1 cells to map genome-wide binding positions of the oncofusion protein. Of the available antibodies (AML: 134, 135 (Diagenode), AML (Cell Signaling), AML (Calbiochem), ETO (Calbiochem), ETO (Santa Cruz)) the one recognizing AML from Calbiochem and ETOsc from Santa Cruz worked best in ChIP. The AML1 Calbiochem antibody was subsequently used in ChIP-chip using a promoter-array and NimbleGen technology. Binding results of the ChIP-chip could be verified using directed ChIP in Kasumi-1 cells and another cell line that expresses AML1-ETO (SKNO-1). To obtain more ChIP-chip and ChIP-seq grade antibodies we again collaborated with partner Diagenode to design and generate ~10 more AML1-ETO antibodies. Ultimately, this led to the identification of one ChIP-seq grade antibody (see below) recognizing the AML1-ETO fusion point (A706 from Diagenode) and three ChIP-seq grade antibodies (see below) recognizing the ETO moiety (A710 and A711 from Diagenode, ETOsc from Santa Cruz). Cells and cellular model systems In the initial phase of Epitron available antibodies that could recognize PML-RARa and AML1-ETO had only limited success in ChIP (and ChIP-chip) assays. Therefore we tagged both PML-RARa and AML1-ETO and transduced the constructs into the U937 cell line. For this the Ty1 tag was used, which is recognized by the BB2 antibody. This antibody had already been successful in ChIP and ChIP-chip assays for other Ty1 tagged proteins in the lab of partner 4. The vectors for transduction of U937 cells were generated and cell lines expressing Ty1-PML-RARa and TY1-AML1-ETO were established. These cell lines were used for performing ChIP assays with promising results. In addition to only using one antibody (BB2), we also explored the possibilities of performing a double ChIP using a combination of the BB2 antibody and an antibody that recognizes the oncofusion protein to get an even higher signal/background ratio. However, these experiments did not yield good results. In addition to the oncofusion proteins the individual protein partners were planned to be tagged in a similar fashion, to explore and compare the binding profile of the individual proteins with the oncofusion protein. However, due to the wealth of data that came along with the establishment of the ChIP-seq technique, these efforts were discontinued. In addition to transduction of tagged PML-RARa in U937 cells, the fusion protein was also

tagged with Ty1 and ER for infection using retroviruses into mouse hematopoietic progenitors in a collaboration with partner Hugues de The. The constructs were made in the lab of partner 7 and the infection of haemopoeitic progenitors took also place there. APL blasts were collected, crosslinked and send for ChIP to the lab of partner 4. Unfortunately, the cell amounts were not sufficient to go into ChIP-seq analysis. New experiments with increased number of cells have in the meantime been initiated and are ongoing. Other cell types that were used in our studies included patient derived cell lines with translocations such as NB4 (PML-RAR) and SKNO-1 and Kasumi-1 (AML1-ETO), zinc inducible oncofusion protein U937 cell lines (UPR and U937AML1-ETO) and blast samples from patients harboring the t(15;17) or t(8;21) translocation. With the establishment of the ChIP-seq technique, various model cell systems, patient blasts and the identification of ChIP-seq grade antibodies two major studies were undertaken, one on the oncofusion protein PML-RARa and one on AML1-ETO. Genome-wide PML-RAR binding in APL Acute promyelocytic leukemia is a distinctive subtype of acute myeloid leukemia (AML) that accounts for approximately 10% of all AML cases. The disease represents a highly malignant form of leukemia with high bleeding tendency and a fatal course of only few weeks. The main diagnostic feature of APL is an aberrant chromosomal translocation that juxtaposes the PML gene on chromosome 15 and the RARα gene on chromosome 17. The resultant chimeric protein, which is found in over 95% of human APLs, retains the DNA binding and ligand binding domains of RARα and the multimerization domain of PML. All-Trans Retinoic Acid (ATRA) and Arsenic Trioxide (ATO) are the two most important drugs in clinical use for the treatment of early diagnosed APL. Both ATRA and ATO degrade the PML-RARα fusion protein by acting on the RARα and PML moieties, respectively. ATRA mainly degrades the protein through proteosome mediated pathways and caspases, while the ATO induced degradation is initiated through sumoylation of the PML moiety. Many mechanisms have been proposed for PML-RARα function, including homodimerization, oligomerization, interaction with RXR, expanded DNA binding affinity and recruitment of a wide spectrum of epigenetic enzymes based on studying a few genomic regions, mostly the promoter of the RARβ gene. In this study, we set out to obtain a comprehensive genome-wide view of the molecular actions of the PML-RARα protein. Using genome-wide ChIP-seq analysis and antibodies recognizing PML (H238, Santa Cruz) and RARa (A704, Diagenode) we identified high confidence PML-RARα binding sites in the leukemic model cell line NB4 and in primary blasts of two APL patients. We found co-localization of PML-RARα with RXR to the vast majority of these binding regions, including genes important in hematopoietic differentiation such as SPI1 (PU.1), GFI1 and RUNX1, ATRA binding proteins such as RARα and RARβ and many genes involved in epigenetic regulation. In addition, genome-wide epigenetic studies revealed that treatment with pharmacological doses of all-trans retinoic acid (ATRA) induces changes in H3 acetylation, but not H3K27me3, H3K9me3 or DNA methylation at the PML-RARα/RXR binding sites or at nearby target genes suggesting that histone acetylation status is closely intertwined with oncofusion protein presence. In addition, we performed genome-wide profiling of RNAPII occupancy and defined nearly 2000 ATRA up or down-regulated genes. To our surprise only 10% of both the up- as the down-regulated genes in all these studies were proximal to PML-RARα/RXR peaks, while the majority of ATRA affected genes where localized at greater distance from these binding sites. Together, our results suggested that PML-RARα/RXR functions as a local chromatin modulator and that specific recruitment of histone deacetylase activities to genes important for hematopoietic differentiation, RAR signalling and epigenetic control is crucial

to the transforming potential of PML-RARα/RXR. These data were published in the beginning of 2010 (Martens et al., Cancer Cell, 2010). Genome-wide AML1-ETO binding in AML Since the publication of the PML-RAR manuscript our analysis focussed on another translocation, t(8;21). The t(8;21) translocation is present in approximately 10% of all de novo AML cases, and results in the expression of the AML1-ETO (RUNX1-RUNX1T1) oncofusion protein. Expression of the AML1-ETO oncofusion protein in hematopoietic cells results in a stage specific arrest of maturation and increased cell survival, predisposing cells to develop leukemia. At the molecular level RUNX1 (AML1) represents a DNA-binding transcription factor required for hematopoiesis, while ETO acts as a co-repressor molecule. The t(8;21) translocation replaces the transactivation domain of RUNX1 (AML1) with the almost complete ETO protein, thereby converting an essential transcriptional activator into a strong repressor. In this study, we analysed the genome-wide DNA binding pattern of AML1-ETO in AML cell lines and in primary AML blasts. Using Kasumi-1 cells and SKNO-1 cells we established the genome-wide profile of AML1-ETO using one antibody (A706, Diagenode) recognizing the AML1-ETO fusion point and three antibodies (A710 and A711, Diagenode; ETOsc, Santa Cruz) recognizing the ETO moiety. In addition we determined genome-wide binding of AML1-ETO using the A706 antibody in 3 blasts from patient with t(8;21). We could demonstrate that AML1-ETO preferentially binds regions that contain RUNX1/AML1 and ETS core consensus sequences and that the AML1-ETO binding sites invariably consist HEB and partially CBFβ, RUNX1/AML1 as well as of ETS factors such as ERG and FLI1. Subsequent analysis of ERG in t(8;21) cells revealed cell type specific ETS factor binding and preferential AML1-ETO binding to the cell type specific ERG binding sites. In addition, we uncovered that ERG binding correlates with the 'active' histone acetylation mark. Together our results revealed that ERG could be a pioneering factor for AML1-ETO binding and that AML1-ETO recruits histone deacteylase activities to ERG occupied regions. A manuscript (Martens et al., see below) was prepared with the results of this analyses and is currently as a revised version under review. PML-RARa and AML1-ETO comparison Comparing the PML-RARα and AML1-ETO binding profiles revealed many common genomic targets, amongst which the hematopoietic master regulators SPI1 and RUNX1. Apart from this several other molecular similarities could be uncovered. First, both oncofusion proteins form oligomeric complexes as an effect of the multimerization properties of the fusion partners PML and ETO. Consequently, the oligomeric complex can target DNA binding templates that contain multiple consensus sequences and thereby deviate from parental protein binding, although the DNA binding domain and hence cis-acting sequence recognition is not changed. Secondly, both oncofusion proteins have a protein partner, HEB and CBFβ that bind to the ETO and AML1 moiety of AML1-ETO, respectively, and RXR that binds the RAR moiety of PML-RARα. Third, our study showed almost exclusive binding of AML1-ETO and PML-RARα to regions occupied by ERG. As previously the ETS factor SPI1 has also been reported to interact with PML-RARα (Wang et al., 2010), these results indicate that both oncofusion proteins are targeted to and could potentially interfere with ETS factors. Finally, our observation that PML-RARα recruits histone deacetylase activities could be extended to AML1-ETO, revealing that both oncofusion proteins recruit histone deacetylase activities to their binding sites. It is tempting to speculate that other oncofusion proteins might also share many of these features or, vice versa, that any protein that is altered such that it confers these four properties has the potential to transform cells. Still, many targets of AML1-ETO and PML-RARα are not shared and our results suggest that ERG might be an

important determinant for this AML subtype specific oncofusion protein binding. These differences in ERG binding regions between PML-RARα and AML1-ETO might account for the specific block of differentiation and features of the diseases. Future analysis of both these common and specific aspects of various AML subtype can be expected to yield further insights on how to therapeutically eradicate these cancer cells. Identification of novel drug targets. Our analysis on genome-wide binding of PML-RARa and AML1-ETO uncovered many new potential targets. First we uncovered that the oncofusion proteins AML1-ETO and PML-RARa recruit genome-wide HDAC activities, revealing that all binding sites are potential HDACi targets. Indeed, HDAC inhibitors could be used as drugs to target these sites as confirmed by several studies that have been conducted to examine the effects of HDAC inhibitors on global and local H3 and H4 acetylation level (see below). In addition, as most oncofusion protein binding events are suspected to be linked to alterations in gene regulation, all oncofusion protein target genes are candidate drug targets. To narrow down these targets a focus could be made on common oncofusion protein binding sites/gene targets or even common oncofusion proteins/ERG binding sites. However, as differences in ERG binding regions between PML-RARα and AML1-ETO might account for the specific block of differentiation and features of the diseases it might also be worthwhile to include genes that are specifically targeted by an oncofusion protein. Finally, our work on AML1-ETO showed that ERG and maybe other ETS factors could be potential drug targets in AML therapy. Analysis of HDAC inhibitors To examine the genome-wide effects of HDACi on histone acetylation we profiled histone H3 and H4 acetylation levels in a variety of cell lines (U937, NB4, Kasumi-1) before and after treatment with HDAC inhibitors such as SAHA and MS275. The results of these experiments are still being analyzed in collaboration with the Altucci lab. Methylome analysis. Initially, we performed MeDIP analysis coupled to microarray hybridization as a technique to examine global DNA methylation changes. Analysis has been performed using DNA from an AML patient, CD34+ and NB4 cells using a combination of MeDIP and microarray analysis (MeDIP-ChIP). The microarray used comprised selected regions from the human genome that are of potential interest for the Epitron consortium. The results showed that the DNA methylation pattern of AML and CD34+ cells is more comparable then that of the NB4 cell line in comparison to the AML cells. However, still numerous differences between AML and CD34+ cells could be detected. This analysis was stopped upon the arrival of next generation sequencing (NGS) methods. Although coupling of these NGS techniques with ChIP worked immediately for global ChIP analysis, it needed extensive optimization for global MeDIP based analysis as the high levels of DNA methylation at repeats significantly disturbed the analysis of the non-repetitive part of the genome. Therefore partner 4 established a method based on an MBD column to asses global DNA methylation called MethylCap-seq. This method was established under the umbrella of the CancerDip consortium. Within Epitron MethylCap-seq was performed using t(8;21) blasts that were partioned based on CD34 expression: both the CD34+ as well as the CD34- t(8;21) cell population were analyzed. In addition, normal CD34+ cells were included in our analysis, as these cells represent the normal counterpart of the t(8;21) leukemic cells. Although data is available, analysis is still in progress and expected to be submitted for publication later this year.

List of selected publications:

Martens JH, Brinkman AB, Simmer F, Francoijs KJ, Nebbioso A, Ferrara F, Altucci L, Stunnenberg HG. PML-RARalpha/RXR Alters the Epigenetic Landscape in Acute Promyelocytic Leukemia. Cancer Cell. 2010 Feb 17;17(2):173-85.

Hatzis P, van der Flier LG, van Driel MA, Guryev V, Nielsen F, Denissov S, Nijman IJ, Koster J, Santo EE, Welboren W, Versteeg R, Cuppen E, van de Wetering M, Clevers H, Stunnenberg HG Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells.. Mol Cell Biol. 2008 Apr;28(8):2732-44. Epub 2008 Feb 11.

Smeenk L, van Heeringen SJ, Koeppel M, van Driel MA, Bartels SJ, Akkers RC, Denissov S, Stunnenberg HG, Lohrum M. Characterization of genome-wide p53-binding sites upon stress response. Nucleic Acids Res. 2008 Jun;36(11):3639-54. Epub 2008 May 12

Welboren WJ, van Driel MA, Janssen-Megens EM, van Heeringen SJ, Sweep FC, Span PN, Stunnenberg HG. ChIP-Seq of ERalpha and RNA polymerase II defines genes differentially responding to ligands. EMBO J. 2009 May 20;28(10):1418-28. Epub 2009 Apr 4.

1.5 PARTNER 5 - UVIGO:

Our proposed contribution to EPITRON objectives was the generation of new epigenetic drugs through three complementary approaches: a) structure-based design of inhibitors of HDACs, HAT, HMT and other epigenetic proteins based on the existing structural information: apo- and holo-crystal structures, the models of the interactions of the proteins with bound ligands (trichostatin, SAHA, AdoHCy…) and the suggested mechanism of ligand inhibition; b) generation of focused libraries inspired by validated natural product inhibitors ["trapoxin-trichostatin chimeras" (for HDACi's), analogs of psammaplins (HDACi's) and dimers (DNMTi's), and of curcumin, garcinol and anacardic acid (HATi's), for example]; c) virtual screening (VS) to sample the virtual library marketplace of drug-like molecules (with the conservative absorption and permeation filtering of Lipinski's rule of five) using flexible ligand docking and ligand scoring, clustering, selection of compounds and focused library synthesis. Of particular interest was the generation of class- and subtype-selective substrates, in order to understand the basis of their selectivity and dissect their function. The comprehensive drug design and synthesis protocol will require iterative cycles of compound selection and optimization to improve pharmacological profiles, including ADME, and continuous validation with the appropriate biochemical, biological and animal assays. Our group has taken the lead of the following tasks: Task 6.1. Modeling of the protein-ligand complexes In order to understand the nature of the ligand-enzyme interactions and the mechanism of epigenetic enzyme inhibition, we have constructed the following models: a) a model for HDAC8 based on the published crystal structure, and its complex with inhibitors UVI5000 (psammaplin A) and indole derivative UVI5008; b) a model of DNMT built by homology and its complex with UVI50008; c) a model of SIRT1 and SIRT2, and an structural proposal for the inhibition of EX527, a potent inhibitor of the sirtuins. Task 6.2. Design and synthesis of derivatives of existing validated inhibitors Within this topic, we have focused on the structure of epigenetic inhibitors that have been described previously in the literature. Using these scaffolds as validated structures, synthetic chemistry efforts have yielded several different classes of HDAC inhibitors, as detailed below: a. A collection of TSA-based HDAC inhibitors, in which the chiral center of trichostatin A has been replaced by the isoelectronic N-methyl (or N-H) derivative. Enamido-TSA analogues have been prepared using as the key step a copper-catalyzed cross-coupling between the benzamides and the corresponding alkenyliodides. Given the difficulties encountered in the saponification of these conjugated enamides, we decided to use instead the non-conjugated derivatives. However, the difficulties on handling and purifying these compounds with hydroxamic acid functional groups led us to shift to other Zn-chelating HDACis. b. A collection of natural-products based inhibitors of the cyclostelletamine family showing class and subclass IIa HDAC selectivity. A collection of macrocyclic bis-pyridinium salts, including some of the natural products, has been prepared using as key step the ring-closing metathesis reaction followed by the hydrogenation of the generated double bond (which was obtained as a mixture of E/Z isomers). The inhibitory activity was determined by partner 6, who found that open-chain derivatives showed more potent biological activities than the macrocyclic natural-products. Furthermore, enzymatic and functional assays have indicated the HDAC1-selectivity of one analogues and the HDAC subclass IIa-selectivity of another inhibitor. These bis-pyridinium dienes can be considered atypical HDAC inhibitors since they do not contain the classical Zn+2 chelating groups, and therefore are first-in-class inhibitors. In order to

confirm their intrinsic activity and improve the potency, we decided to prepare bis-byridinium dienes having the classical Zn+2 chelating groups, including the variation in the chain length. Only series A and B were prepared although upon attempted deprotection of the hydroxamic acid, the corresponding methyl esters were instead obtained. On the other hand, the synthesis of analogues B1 and B2 was based on the cross-metathesis of pyridinium alkenes and unsaturated thioesters. The routes to the other analogues met with failure at some of the steps of the proposed sequence. c. Synthesis of novel anacardic acid amides, potential histone acetyltransferase (KAT) inhibitors We have continued with the preparation of benzamides related to anacardic acid amide CTPB with a 4-cyano-3-trifluoromethyl-phenylamine and alkyl chains of defined length starting from 2,6-dihydroxybenzoic acid. Two series were synthesized: 1) analogues with a primary alcohol group attached to a chain of 3 to 6 carbon atoms; 2) analogues that have incorporated al alkenyl group or aryl substituents. The strategies are slightly different, since in the former case the hydroxyalkyl substituent replaces the triflate using a Suzuki reaction, whereas in the second this step takes place after the preparation of the amide. Task 4. In silico ADMET and experimental PK/CYP induction values of UVI5008. The ADME properties of lead compound UVI5008 and derivatives have been estimated using freely available software. Moreover, we have carried out experimental in vitro/in vivo ADMET analysis of lead compound UVI5008 in the CEREP company. The evaluation included two assays: 1) PK evaluation of UVI5008 in mouse using three administration routes, according to the following protocol: - Intravenous (1 mg/kg bolus), intraperitoneal (5 mg/kg), and peroral (5 mg/kg as suspension) administration of compound UVI5008 to mice. - Withdrawas of blood samples from jugular vein at 8 time points (up to 24 h) after administration. - Determination of plasma levels of parent compound measured with an LC/MS method. - Quantitative plasma bioanalysis: o Cmax (nM): peak plasma levels o tmax (h): time required to reach peak plasma levels o AUC: Area under the plasma levels time curve o Vd (L/Kg): volume of distribution o T1/2 (h): plasma half-life o Clearance CL p values (L/h/kg) o F (%): bioavailabilities (%) A very rapid loss of MS signals corresponding to either the disulfide UVI5008 or its thiol derivative was detected. Since it was anticipated that the disulfide would be rapidly reduced in the mice due to the action of glutathione reductase, the analysis was carried out monitoring the mass corresponding to the glutathione conjugate. . 1. The PK parameters for the glutathione conjugate in IV dosed animals had to be calculated using extravascular input (i.e. non-IV dosing) model because this glutathione conjugate is not the dosed compound, rather a derivative of the dosed compound. 2. The concentrations of the glutathione conjugate can only be expressed as peak area but not ng/mL because there is no standard for this conjugate to make a calibration curve. The common non-IV parameters are: Tmax (min), Cmax (ng/mL), T1/2 (min), AUCINF (min*ng/mL), and AUClast (min*ng/mL). In the case for this glutathione conjugate, all ng/mL has to be expressed as peak area. The bioavailability for the conjugate was calculated as a relative bioavailability. The absolute bioavailability is not calculable because the conjugate itself was not an IV dosed compound. The results confirm that the disulfide UVI5008 is a pro-drug that gets rapidly transformed into the glutathione adduct (MW: 661.55). This derivative incorporates one unit of the derived thiol in the form of a mixed disulfide. 2) ADME-Tox Cytochrome P450 inhibition of UVI5008 (1 x 10-5 M) using human liver microsomes as an estimation of in vitro metabolism. Peak areas corresponding to the metabolite of each substrate were recorded. The percent of control activity was calculated by comparing the peak area obtained in the presence of the test compound to

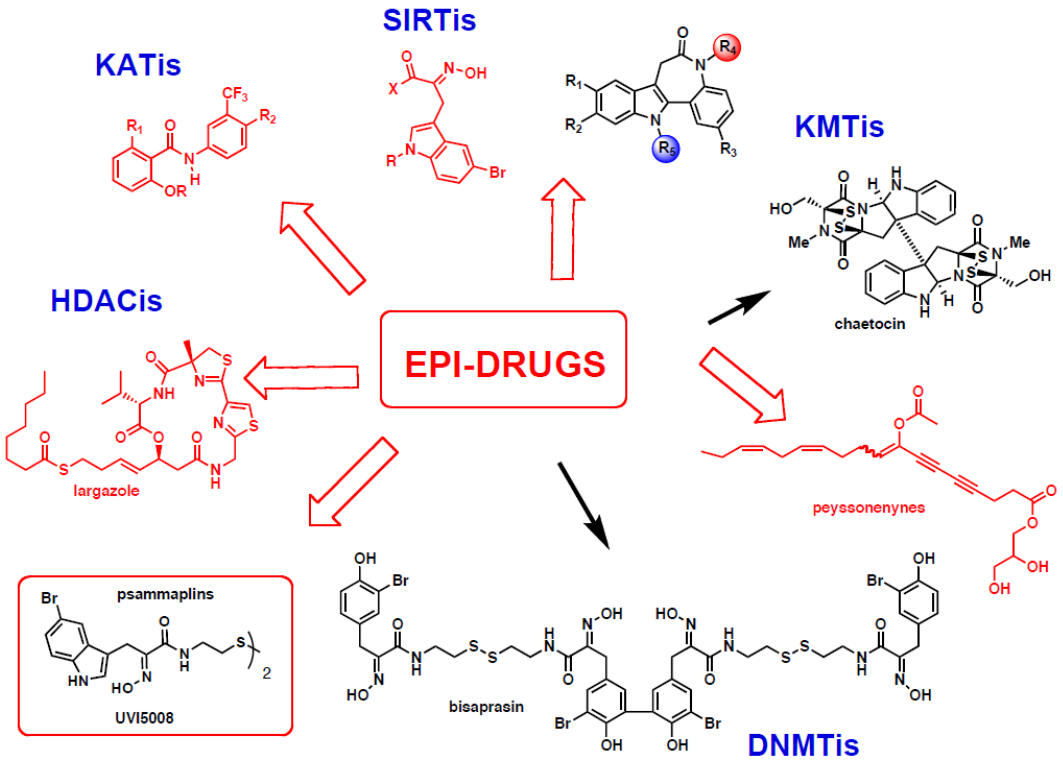
that obtained in the absence of the test compound. Subsequently, the percent inhibition was calculated by substracting the percent control activity from 100 for each compound. IC50 values were determined by non-linear regression analysis of the concentration-response curve using Hill equation curve fitting. a) CYP1A inhibition (HLM, phenacetin substrate): 36% b) CYP2C8 inhibition (HLM, paclitaxel substrate): 55% c) CYP2D6 inhibition (HLM, dextromethorphan substrate): 55% d) CYP3A inhibition (HLM, midazolam substrate): 40% Task 6.6. Structural optimization of psammaplin A as HDAC inhibitor Upon modification of the nature of the different components or building blocks a collection of psammaplin A analogues has been prepared that contain the following modifications: a) a chain of variable lenght (between 2 and 6 methylene groups between amide and disulfide functional groups) b) an all-carbon connector joining both oxime amides c) protecting groups at the oxime d) other aryl and brominated aryl rings e) in addition, the monomeric alcohol and the methylsulfide would provide insights into the mechanism of HDAC8 inactivation by psammaplin A. Structure-activity relationships (SAR) principles from this study are the following: a) the chain lenght of the natural product is already optimal: greater lengths lead to less active inhibitors b) the all-carbon analogue is inactive, implying that the sulfur is key to the inhibitory activity c) protection of the oxime, at least with bulky groups, abolishes activity d) bromoindol analogues show an increase on activity except the C4-bromoindol e) the monomeric alcohol and the methylsulfide showed also reduction on their activity, which together with the findings in b) indicates that the thiolate must be the zinc-chelating group, and therefore psammaplin A is a pro-drug that gets activated in the cells (most likely via glutathione addition). The mechanism should be similar to that proposed for the depsipeptide FK228. Based on the promising results with the psammaplin A scaffold, we carried out a synthetic program aimed to increase the potency as antitumor agent. Notably, among the structures prepared, the analogues with haloindoles proved to be the most potent. The synthetic sequence devised to prepare the HDAC inhibitors with bromoindol structure uses as critical step an inverse demand hetero Diels-Alder reaction between indol and the nitrosovinyl compound obtained in situ, followed by ring opening of the oxazine (or a Friedel-Crafts/trapping/ring opening scheme). Once the oxime group is protected, the construction of the dipeptides follows the same reactions explained with the parent psammaplins. We have establishing SAR studies after the synthesis and biological characterization of analogs which preserve the requisite structural features considered responsible for the HDAC inhibitory activity (Zn-chelating group, chain and rim-located group) that contain the following modifications: a) Incorporation of the remaining halogens at position C5 of the indole ring following the same methodology as for the parent system. b) Incorporation of alkoxyl groups at C5 instead of the bromine atom. c) Influence of the oxime in the biological activity. d) Modification of the terminal group: o-aminoacetanilide, hydroxamic acids and glycine methyl esters. e) N-alkylderivatives of the indole ring. The main findings of this section, of interest for the ongoing structure-activity relationships (SAR) study are the following: a) Substitution of disulfide bridge by hydroxamic acid in psammaplin A led to a compound that produced apoptosis in the same manner of SAHA. b) For the indol derivates, the optimal chain length for a good activity was two carbon atoms. Preliminary results showed that the isomer C-6-Br could show selectivity for HDAC class II whereas isomers C-5-Br and C-4-Br showed inhibitory activity for both HDAC class II and I. c) Only the N-methylated indole ring produced apoptosis in the same manner as UVI5008. The more bulky analog (p-bromobenzylo) did not induce apoptosis. d) The bromoindol analogues show

an increase on activity except the C4-isomer. e) The oxime functional group is not required for the HDACi activity. f) Alkoxyl groups at C5 are tolerated. Additional SAR studies on UVI5008 have focused on the replacement of the bromine at C5 by heteratoms/heterocycles. We succeeded on the incorporation of nitrogen derivatives at position C5 of the indole ring using Pd-catalyzed cross-coupling. However, the subsequent Friedel-Crafts reaction gave unexpectedly mixtures of substitution products at the C3 and C4 positions, and therefore this series was not pursued further. Task 6.7. Design and synthesis of class I-selective HDAC inhibitors: Cyclic depsipeptides, including the synthesis of largazole. The depsipeptide largazole was isolated from a Symploca species in Key Largo (Florida) and characterized as a cyclic depsipeptide (J. Am. Chem. Soc. 2008, 130, 1806-1807). It was shown to inhibit the growth of highly invasive transformed human mammary epithelial cells (MDA-MB-231) (GI50 7.7 nM) vs non transformed murine mammary epithelial cells (NMuMG) and fibroblastic osteosarcoma U2OS cells (GI50 55 nM, LC50 94 nM) vs nontransformed fibroblasts NIH3T3. There are some structural similarities between psammaplin A and largazole in what refers to their nature as pro-drugs. In the case of largazole, the action of an esterase will release the butenylthiol group, which is isosteric with the ethenylthiolamide of psammaplin A. Therefore, the potent and tumor-selective activities of largazole are related to its HDAC inhibitory activity. We have carried the preparation of largazole and analogues. The synthesis of largazole and those analogues that differ in the substituent at the dihydrothiazole unit (at the C7 position) entailed as key step the acyclic ring-closing metathesis after the construction of the macrocycle. The remaining steps are classical peptide bond constructions, once the different precursors have been made. The largazole derivative with a bis-thiazole system was constructed using a different strategic bond construction. The key step is a PyBOP/DIPEA-induced lactonization with the butenylthiol protected as trityl derivative, deprotection of this group was and formation of the octanoyl ester. Task 6.8. Synthesis of immobilized psammaplin A derivatives. Considerable time and effort has been devoted to the immobilization of lead compound UVI5008 into Sepharose beads. Two potential points of attachment are the oxime and the indol ring. Functionalization at the oxime group was considered optimal for interaction with the enzymes responsible for the SIRT inhibition activity since this functional group was most likely not required for the HDAC activity of UVI5008. Alternatively, the attachment of the Sepharose to the position of the bromoindole was considered not to affect functionally its HDAC inhibitory activity. The aminopropylphenol group was incorporated at the C5 position, and the general tetrapeptide disulfide dimer was built. However, we have faced problems with the selectivity of the deprotection step, which under all conditions tested resulted in product degradation. Considering that the increased charge density by the indole ring could be responsible for the degradation of the structure under acidic conditions, a 2-aminoethylgroup was used instead, and the required structure of the UVI5008 derivative was completed. As before, the deprotection of the O-trityl and N-Boc groups faced low yields and degradation of the products. As alternatives, the use of other protecting groups such as O-SEM and N-Teoc was also considered, together with other substrate that does not incorporate the offending oxime group but our efforts have been so far unsuccesful. Task 6.9. Design and synthesis of methyltransferase inhibitors DNMT inhibitors a) Total synthesis of bisaprasin depicts the proposed synthesis of bisaprasin, a natural product isolated from the sponge Pseudoceratina purpurea, with reported DNMT and HDAC activities. Our approach to bisaprasin is similar to that described for the psammaplins and requires the

prior construction of the biaryl bond. Whereas the construction of the carbon skeleton has been optimized, conditions for protecting-group-free synthesis could not be developed. Both the oxime and the phenol units of the monomers have been protected with several derivatives (MEM acetals, benzyl ethers, silylethers, Fmoc, allyl ethers). Regrettably, all conditions attempted for the deprotection of these groups at all positions have proven unsuccesful thus far and under certain conditions the number of these protecting groups has been reduced to four. The route is shorter than the one completed, which provide the tetra-OMOM/OAllyl/OBn-protected bisaprasin, since it minimizes the use of protecting groups and moreover uses as first step a biomimetic phenol oxidative coupling induced by vanadyl trifluoride. To perform the last step of the synthesis we tested different methods of deprotection depending on the type of protecting group: - O-MOM. We used a variety of methods (2M HCl in Et2O, conc. HCl, HCl in the presence of NaI, TMSI, TFA, PPTS in tBuOH, bromocatecholborane, Amberlyst) all of which led to degradation of the reactant. - O-Allyl. For deprotection of the allyl group we attempted different reagents, namely Pd(OAc)2, Pd(PPh3)4 in HCO3H/Et3N DDQ, ZrCp2Cl2, and observed different degradation products. - O-Bn. Deprotection of the benzyl groups with BBr3 in thioanisole, TFA with hexamethylbenzene, BCl3·SMe2, and Pd(OH)2 in H2 atmosphere led to mixture of products and no traces of bisaprasin was detected by analysis of the crude 1H-NMR spectra. Although the use of alternative protecting groups was explored, they are less useful due to their lower reactivity in the incorporation (THP, trityl and TBDPSi), or in the deprotection steps (SEM, using HCl or TBAF). b. Total, enantioselective synthesis of the peyssonenynes. The monoacyl glycerol fatty acid derivatives peyssonenynes are natural products isolated from the brown algae Peyssonelia caulifera with reported DNMT inhibitory activity. We have synthesized the four stereoisomers of the peyssonenynes. After exploring different strategies, we found that the most convergent one involving Cadiot-Chodkiewicz coupling of iodoalkyne and terminal alkyne was successful and afforded the entire skeleton of the desired target. The acetal group was deprotected to furnish the natural products. We have also determined their geometry using the pulse sequence EXSIDE that measures long-range 13C-1H coupling constants, and have identified unequivocally peyssonenyne A as the E isomer and peyssonenyne B as the Z isomer (this is the order of elution in reverse-phase HPLC). Moreover, considering that the peyssonenynes might also be a pro-drug, which undergo hydrolysis of the enolacetate followed by addition of a nucleophile in a Michael-type fashion, we have designed and synthesized a series of analogues that are not based on fatty acid, but phenyl, naphthyl and 3-thienyl groups, and have exchanged the glycerol by a tert-butyl ester. Given the fact that the small amounts of the peyssoenynes isolated from Peyssonelia precluded the determination of the absolute configuration of the chiral center and the geometry of the enolacetate, the four stereoisomers have been prepared. The determination of the geometry is challenging, since NOE experiments in NMR have been inconclusive thus far and we are trying to develop an spectroscopic method to confirm the geometries using the values of long-range 13C-1H coupling constants. Synthesis of KHMT inhibitors Chaetocin has been identified as a potent inhibitor of the histone methyltranferase SU(VAR) 3-9 after screening of a library of 2900 compounds, and found to display promising properties for the treatment of myeloma, both in vitro and in vivo. We embarked on a program aimed to the preparation of the hexahydro [2,3-b]pyrroloindole core from the bis-protected tryptophan derivative. Starting from D-trytophan, the open-chain dipeptide was obtained in good yields. The bromide, obtained by stereoselective NBS

cyclization was deprotected on the Fmoc group, which was accompamied by cyclization. Methylation of the amide, followed by dimerisation using a Co(I) compound afforded the unprotected bis-indolizine in good yields. The dimeric diketopiperazine is the most advanced intermediate in our synthesis so far and it contains the carbon skeleton of the chaetocin. After the preparation of the protected monomer, the functionalization of the diketopiperazine ring was carried out via radical formation with NBS and the radical initiator V-70 in CCl4. Without purification, the residue after solvent removal was treated with several nucleophiles: (a) MeOH, (b) KSAc, (c) TrSH and (d) xanthate. In all cases, complex mixtures of products were obtained. After HPLC purification, some of the componentes could be isolated and characterized as the structures shown, but the yields were very low. As model systems for the proposed construction of homo and heterodimers of the pyrroloindoline unit, we selected the diketopiperazine alkaloids WIN 64821 and WIN 64745 first isolated from Aspergillus sp. cultures by the group of Barrow at Sterling Winthrop Pharmaceuticals in 1993, which are potent sustance P antagonists for the human neurokinin-1 and the cholecystokinin B receptors, potential therapeutic targets for the treatment of arthritis, asthma or inflammatory bowel disease. We have devised a versatile synthetic route leading to WIN 64821 and WIN 64745, a strategy that enables the preparation of the C1-non-symmetrical congeners WIN 64745 from the same intermediate. Following the same approach we have proceeded to prepare the remaining congener, which we have named (+)-asperdimin, a compound isolated from extracts of Aspergillus niger in 2004 and shows antiviral activity against certain viruses (flaviviruses and picornaviruses) by inhibiting a nucleotide segment or internal ribosomal entry site (IRES) that guides the host-dependent viral polyprotein synthesis. The proposed structure of the natural product, although correct in the atom connectivity, was not compatible with the spectroscopic and optical rotation data. A synthetic effort was required to secure the correct stereostructure beyond any doubt, which made necessary the synthesis of some stereoisomers. Task 6.10. Synthesis of sirtuin inhibitors. This program was initiated to develop epigenetic modulators of the Sirtuin family of histone deacetylase inhibitors based on the 5-substituted indole core and on the kenpaullone scaffold. Achievements have been the following: a) A series of 5-substituted indoles (simpler analogs of UVI5008) functionalized as esters or carboxylic acids that with a heteroatom/heterocycle at the C5 position (benzylamine, pyrrolidine, piperazine and morpholine). b) A small library of paullones prepared by Suzuki reaction of previously prepared aniline pinacol boronates that have different substituents at several of the skeletal position in order to derive SAR correlations and optimization of their Sirt inhibitory potential. Task 6.11. Generation and Validation of Histone Lysine Methyltransferase and Lysine Demethylase Inhibitors A reported inhibitor of AdoHcy hydrolase, 3-deazaneplanocin A (DZNep) depletes cellular levels of the PRC2 components and inhibit the associated H3H27 demethylation (Genes Dev. 2007, 21, 1050), inducing efficient apoptotic cell death in cancer cells but not in normal cells. We have completed the synthesis of the positional isomers 3-(DZNep) and 7-deazaneplanocin A. Following the same scheme, analogues having different heterocycles and amine substituents have been made and the reactions succesfully optimized. The synthesis of 7-deazaneplanocin A and analogues was achieved using two main reactions: a Mitsunobu reaction and a functionalization of 6-chloro-7-deazapurine ring with secondary amines. 7-Deazaneplanocin A and the analogues substituted at the C6' position with amines such as piperidine, morpholine, methylpiperazine and aniline were prepared Task 6.12. Virtual ligand screening (VLS) to sample virtual libraries of drug-like

molecules using flexible ligand docking and ligand scoring using the crystal structure of the JMJD2A protein (a jumonji-containing histone-lysine demethylase HDMs), selection of leads and focused library generation by chemical synthesis Initially, Task 6.3 (VLS) was proposed for HDAC and DNMT inhibitors, but was not pursued due to the already existing, validated hits obtained from the natural products reservoir. Nevertheless, VLS has been later incorporated in the project to search for scaffolds directed towards the inhibition of Histone Demethylases and therir validadtion as novel drugable targets for epigenetic control of cancer. Given the lack of lead compounds until very recently (pyridine 2,4-dicarboxylic acid as a α-oxoglutarate mimic is a good inhibitor, as is SAHA and other hydroxamic acids) we planned to discover histone demethylase inhibitors of the Jumonji family, by virtual ligand screening, in collaboration with partners #10 and #14. Taking the JMJD2A crystal structure as a lead (a Ni(II)-Zn(II)-containing complex bound to tri-, di-, and monomethyl forms of H3K9 and the trimethyl form of H3K36; Nature 2007, 448, 88) we have screened, in collaboration with partner #9, drug-like chemical libraries using docking of flexible ligands and classification of ligands. The prioritized list was further clustered, and the 67 hit compound were screened by partner #10. FINAL SUMMARY OF ACTIVITIES OF PARTNER #6 As a final consideration, we have demonstrated the validity of the approach to the discovery of novel inhibitors of epigenetic enzymes using the scaffolds of the natural products. Our contribution is summarized in the following scheme, which highlights the main deliverables of our work, grouped according to the enzyme targets as well as to the structures of the leading compounds. Of particular relevance is the discovery of the triple inhibitor UVI5008, a natural product derivative, which has been patented (Derivatives of psammaplin A, a method for their synthesis and their use for the prevention or treatment of cancer, EP20070290253; owned by partners #1, #5, #6; NOVEL DERIVATIVES OF PSAMMAPLIN A, A METHOD FOR THEIR SYNTHESIS AND THEIR USE FOR THE PREVENTION OR TREATMENT OF CANCER, WO2008IB01887; owned by partners #1, #4, #5, #6). Despite being a single chemical entity, UVI5008 inhibits at once three distinct classes of epigenetic enzymes, histone deacetylases, sirtuins and DNA methyltransferases. UVI5008 displays cancer cell-selective activity and activates multiple tumoricidal pathways. This redundancy enables the triple inhibitor to target p53 or TRAIL wild-type and mutant/deficient tumor cells with similar efficiency. The activities of UVI5008 were validated in vitro, in leukemia patients' blasts ex vivo, in human colon cancer xenografts and in primary tumors of genetic mouse models for breast cancer. In keeping with its pharmacokinetics the epigenetic activities of UVI5008 were demonstrated in tumori. This compound is expected to enter Phase I clinical trials.



Summary of the contribution of partner 5

List of selected publications:

Bispyridinium dienes: histone deacetylase inhibitors with selective activities.Pérez-Balado C, Nebbioso A, Rodríguez-Graña P, Minichiello A, Miceli M, Altucci L, de Lera AR. J Med Chem. 2007 May 17;50(10):2497-505. Epub 2007 Apr 21.

Feijoa sellowiana derived natural Flavone exerts anti-cancer action displaying HDAC inhibitory activities.Bontempo P, Mita L, Miceli M, Doto A, Nebbioso A, De Bellis F, Conte M, Minichiello A, Manzo F, Carafa V, Basile A, Rigano D, Sorbo S, Castaldo Cobianchi R, Schiavone EM, Ferrara F, De Simone M, Vietri M, Cioffi M, Sica V, Bresciani F, de Lera AR, Altucci L, Molinari AM.Int J Biochem Cell Biol. 2007;39(10):1902-14. Epub 2007 May 25.

Synthesis of benzamides related to anacardic acid and their histone acetyltransferase (HAT) inhibitory activities.Souto JA, Conte M, Alvarez R, Nebbioso A, Carafa V, Altucci L, de Lera AR. ChemMedChem. 2008 Sep;3(9):1435-42.

Synthesis and biological characterization of the histone deacetylase inhibitor largazole and C7- modified analogues.Souto JA, Vaz E, Lepore I, Pöppler AC, Franci G, Alvarez R, Altucci L, de Lera AR.J Med Chem. 2010 Jun 24;53(12):4654-67.

New anacardic acid-inspired benzamides: histone lysine acetyltransferase activators.Souto JA, Benedetti R, Otto K, Miceli M, Alvarez R, Altucci L, de Lera AR. ChemMedChem. 2010 Sep 3;5(9):1530-40.

Determination of the geometry of acetoxyendiynes and acetoxyenynes by NMR heteronuclear (13)C-(1)H scalar couplings and (13)C NMR chemical shifts. Structural assignment of the oxylipin natural products peyssonenynes A and B.García P, Martín-Pastor M, de Lera AR, Alvarez R.Magn Reson Chem. 2010 Jul;48(7):543-9.

Epigenetic profiling of the antitumor natural product psammaplin A and its analogues.García J, Franci G, Pereira R, Benedetti R, Nebbioso A, Rodríguez-Barrios F, Gronemeyer H, Altucci L, de Lera AR. Bioorg Med Chem. 2011 Jun 15;19(12):3637-49. Epub 2010 Dec 15.

Total synthesis of the proposed structures of the DNA methyl transferase inhibitors peyssonenynes, and structural revision of peyssonenyne B. García-Domínguez P, Lepore I, Erb C, Gronemeyer H, Altucci L, Alvarez R, de Lera AR. Org Biomol Chem. 2011 Aug 22.

1.6 PARTNER 6 - SUNAP:

The contribution of SUNAP has spanned through the three RAs as also corroborated by the publications and collaborations. Within the first RA we have been contributing to the decryption of the role of the epimarks in acute myeloid leukemias with particular focus to the impact of fusion proteins such as PML-RAR and AML.ETO on the epigenome (chromatin mod. and methylation) deregulation. Our contribution has been in a collaborative manner with other partners of the consortium such as HS and SM. As leader of RA2 SUNAP has been performing studies to identify and characterise novel epigenetic drugs against cancer. Our contribution has been the understanding of the mechanism(s) of action of HDAC inhibitors in leukemias. We have addressed this issue in different ways .By knocking down both HDAC1 and 2 (Conte et al. manuscript in preparation) thus defining the specific (non overlapping) signatures of these 2 epi-targets we have addressed the specific HDAC contribution to the action of HDAC inhibitors. The results show that HDAC1 and 2 play a repressive role which is only partially dependent by their enzymatic inhibition, suggesting that additional functions are mainly related to the complexes that they take part of and to non-catalytic domains of the proteins. Interestingly our data indicate specific signatures dependent by HDAC1 or 2 knock-down thus defining specific HDAC1/2 profiles. On a different approach, we have been studying the mechanism of apoptotic action of known HDACis such as entinostat and vorinostat. We have identified cMYC as a key regulator of the cellular response to HDACis in acute myeloid leukemias and characterised the molecular events underlying HDACi MYC regulation in vitro, ex vivo and in patients. A main effort within RA2 has been devoted to the identification in vitro and characterisation in cells of a large number of epidrugs produced within the consortium. The main targets of these epidrugs have been HDACs, SIRTs, HATs, HMTs and KDMs as stated by the number of joint publications. Among all we have identified together with partners HG, HS and AdL a novel triple inhibitor of HDACs, DNMTs and SIRTs that has been patented for its anticancer potential. The relative manuscript in coll. with Matthias Nees (OK group) (for some of the gene expression profiling) is upon submission (Nebbioso et al). Moreover we have been also characterising another class of SIRT inhibitors with anticancer potential. Also in this case we have been characterising the mechanism(s) that underlie the anticancer action (Carafa et al, upon submission). By addressing the issue of epicompounds specificity we could originally demonstrate the action of class II HDAC inhibitors in several models (not only cancer) such as muscle and adipose differentiation (studies have been published in BJ, EMBO Rep and JME). Finally, as part of RA II (WP5) we have been not only testing epidrugs (HDACis and SIRTis) on known stepwise tumorigenesis models, but we have been creating novel tumorigenesis models both CD34+ and amniocyte-based (Miceli et al, upon submission). These models have strengthen the role of MYC in the cellular response to HDACis and may represent novel systems to test the activity of epidrugs on both differentiation and cell death. As red-out of RAII, a part the number of publications, an international patent (WO2008125988A1) has been filled by us and other three partners of the Epitron consortium (HG, AdL and HS). In the patent we report on the synthesis and anticancer action (prevention and treatment) of UVI5008. We also report on the mechanism of action of UVI5008, a novel epigenetic modifier, and show that it inhibits histone deacetylases, sirtuins and DNA methyltransferases. UVI5008 induces cancer cell-selective death in a variety of models and exerts its activities in several human tumor xenografts and genetic mouse models of human breast cancer in vivo. Its anticancer activity involves

activation of death receptors and ROS production in a mutually independent manner. Importantly, UVI5008 targets tumor cells that are wild-type, mutated or deficient for p53, BMF and/or TRAIL with similar efficacies, thus limiting the risk of drug resistance development and maximizing its application spectrum. Within RA3 our main contribution has been in WP10 where we have been identifying first and characterizing later epi-based combo-treatments. Among all, we have identified the mechanistic basis of the anti-leukemia action of chaetocin used alone or in combination with ARA-C (Chaibetal, Nebbioso et al, under revision). Moreover, by testing the action of rTRAIL in ex vivo AML models we have stratified AML samples in base of the resistance and applied gene expression profiling to identify and characterize mechanisms of resistance. We attach some of the publications and the patent. Publications upon submission or submitted have not been attached but, of course, will thank Epitron support.

List of selected publications:

García J, Franci G, Pereira R, Benedetti R, Nebbioso A, Rodríguez-Barrios F, Gronemeyer H, Altucci L, Lera AR. Epigenetic profiling of the antitumor natural product psammaplin A and its analogues.Bioorg Med Chem. 2011 Jun 15;19(12):3637-49. Epub 2010 Dec 15.

Di Bernardo G, Alessio N, Dell'Aversana C, Casale F, Teti D, Cipollaro M, Altucci L, Galderisi U.Impact of histone deacetylase inhibitors SAHA and MS-275 on DNA repair pathways in human mesenchymal stem cells. J Cell Physiol. 2010 Nov;225(2):537-44.

Borbone E, Berlingieri MT, De Bellis F, Nebbioso A, Chiappetta G, Mai A, Altucci L, Fusco A.Histone deacetylase inhibitors induce thyroid cancer-specific apoptosis through proteasome-dependent inhibition of TRAIL degradation. Oncogene. 2010 Jan 7;29(1):105-16. Epub 2009 Oct 5

Martens JH, Brinkman AB, Simmer F, Francoijs KJ, Nebbioso A, Ferrara F, Altucci L, Stunnenberg HG. PML-RARalpha/RXR Alters the Epigenetic Landscape in Acute Promyelocytic Leukemia. Cancer Cell. 2010 Feb 17;17(2):173-85.

Shankaranarayanan P, Rossin A, Khanwalkar H, Alvarez S, Alvarez R, Jacobson A, Nebbioso A, de Lera AR, Altucci L, Gronemeyer H. Growth factor-antagonized rexinoid apoptosis involves permissive PPARgamma/RXR heterodimers to activate the intrinsic death pathway by NO. Cancer Cell. 2009 Sep 8;16(3):220-31

Valente S, Tardugno M, Conte M, Cirilli R, Perrone A, Ragno R, Simeoni S, Tramontano A, Massa S, Nebbioso A, Miceli M, Franci G, Brosch G, Altucci L, Mai A. Novel cinnamyl hydroxyamides and 2-aminoanilides as histone deacetylase inhibitors: apoptotic induction and cytodifferentiation activity. ChemMedChem. 2011 Apr 4;6(4):698-712.

Souto JA, Benedetti R, Otto K, Miceli M, Alvarez R, Altucci L, de Lera AR. New anacardic acid-inspired benzamides: histone lysine acetyltransferase activators. ChemMedChem. 2010 Sep 3;5(9):1530-40.

Nebbioso A, Manzo F, Miceli M, Conte M, Manente L, Baldi A, De Luca A, Rotili D, Valente S, Mai A, Usiello A, Gronemeyer H, Altucci L. Selective class II HDAC inhibitors impair myogenesis by modulating the stability and activity of HDAC-MEF2 complexes.EMBO Rep. 2009 Jul;10(7):776-82. Epub 2009 Jun 5.

Lara E, Mai A, Calvanese V, Altucci L, Lopez-Nieva P, Martinez-Chantar ML, Varela-Rey M, Rotili D, Nebbioso A, Ropero S, Montoya G, Oyarzabal J, Velasco S, Serrano M, Witt M, Villar-Garea A, Imhof A, Mato JM, Esteller M, Fraga MF. Salermide, a Sirtuin inhibitor with a strong cancer-specific proapoptotic effect. Oncogene. 2009 Feb 12;28(6):781-91. Epub 2008 Dec 8. Erratum in: Oncogene. 2009 Feb 26;28(8):1168. Inhof, A [corrected to Imhof, A].

Colussi C, Mozzetta C, Gurtner A, Illi B, Rosati J, Straino S, Ragone G, Pescatori M, Zaccagnini G, Antonini A, Minetti G, Martelli F, Piaggio G, Gallinari P, Steinkuhler C, Clementi E, Dell'Aversana C, Altucci L, Mai A, Capogrossi MC, Puri PL, Gaetano C. HDAC2 blockade by nitric oxide and histone deacetylase inhibitors reveals a common target in Duchenne muscular dystrophy treatment. Proc Natl Acad Sci U S A. 2008 Dec 9;105(49):19183-7. Epub 2008 Dec 1.

García, J., Franci, G., Pereira, R., Benedetti, R., Nebbioso, A., Rodríguez-Barrios, F.,Gronemeyer, H., Altucci, L., Lera, R.d., Epigenetic Profiling of the Antitumor Natural Product Psammaplin A and Its Analogues, Bioorganic & Medicinal Chemistry (2010), doi: 10.1016/j.bmc.2010.12.026

Mai A, Valente S, Cheng D, Perrone A, Ragno R, Simeoni S, Sbardella G, Brosch G, Nebbioso A, Conte M, Altucci L, Bedford MT. Synthesis and biological validation of novel synthetic histone/protein methyltransferase inhibitors. ChemMedChem. 2007 Jul;2(7):987-91

Bontempo P, Mita L, Miceli M, Doto A, Nebbioso A, De Bellis F, Conte M, Minichiello A, Manzo F, Carafa V, Basile A, Rigano D, Sorbo S, Castaldo Cobianchi R, Schiavone EM, Ferrara F, De Simone M, Vietri M, Cioffi M, Sica V, Bresciani F, de Lera AR, Altucci L, Molinari AM. Feijoa sellowiana derived natural Flavone exerts anti-cancer action displaying HDAC inhibitory activities. Int J Biochem Cell Biol. 2007;39(10):1902-14. Epub 2007 May 25.

Manzo F, Nebbioso A, Miceli M, Conte M, De Bellis F, Carafa V, Franci G, Tambaro FP, Altucci L. TNF-related apoptosis-inducing ligand: signalling of a 'smart' molecule. Int J Biochem Cell Biol. 2009 Mar;41(3):460-6. Epub 2007 Dec 28.

Mai A, Altucci L.Epi-drugs to fight cancer: from chemistry to cancer treatment, the road ahead. Int J Biochem Cell Biol. 2009 Jan;41(1):199-213. Epub 2008 Aug 22.

Mai A, Valente S, Rotili D, Massa S, Botta G, Brosch G, Miceli M, Nebbioso A, Altucci L. Novel pyrrole-containing histone deacetylase inhibitors endowed with cytodifferentiation activity. Int J Biochem Cell Biol. 2007;39(7-8):1510-22. Epub 2007 Apr 4.

Mai A, Massa S, Rotili D, Simeoni S, Ragno R, Botta G, Nebbioso A, Miceli M, Altucci L, Brosch G. Synthesis and biological properties of novel, uracil-containing histone deacetylase inhibitors. J Med Chem. 2006 Oct 5;49(20):6046-56.

Pérez-Balado C, Nebbioso A, Rodríguez-Graña P, Minichiello A, Miceli M, Altucci L, de Lera AR. Bispyridinium dienes: histone deacetylase inhibitors with selective activities. J Med Chem. 2007 May 17;50(10):2497-505. Epub 2007 Apr 21.

Souto JA, Vaz E, Lepore I, Pöppler AC, Franci G, Alvarez R, Altucci L, de Lera AR. Synthesis and biological characterization of the histone deacetylase inhibitor largazole and C7- modified analogues. J Med Chem. 2010 Jun 24;53(12):4654-67.

Nebbioso A, Dell'Aversana C, Bugge A, Sarno R, Valente S, Rotili D, Manzo F, Teti D, Mandrup S, Ciana P, Maggi A, Mai A, Gronemeyer H, Altucci L. HDACs class II-selective inhibition alters nuclear receptor-dependent differentiation.J Mol Endocrinol. 2010 Oct;45(4):219-28. Epub 2010 Jul 16.

Altucci L, Leibowitz MD, Ogilvie KM, de Lera AR, Gronemeyer H.RAR and RXR modulation in cancer and metabolic disease. Nat Rev Drug Discov. 2007 Oct;6(10):793-810. Review.

Manzo F, Tambaro FP, Mai A, Altucci L. Histone acetyltransferase inhibitors and preclinical studies. Expert Opin Ther Pat. 2009 Jun;19(6):761-74. Review.

Altucci L, Minucci S.Epigenetic therapies in haematological malignancies: searching for true targets. Eur Cancer. 2009 May;45(7):1137-45. Epub 2009 Apr 5.

1.7 PARTNER 7 - CNRS:

Our expertise in APL, a disease on which a significant part of the EPITRON consortium relies on, has allowed us to provide significant advances in our understanding of the pathogenesis of the disease and the basis for treatment response. We have first demonstrated that the RXRA, the coreceptor of RARA is required for transformation by PML/RARA. The presence of RXR in the PML/RARA complex allows the binding to highly degenerated DNA sites. Moreover, PML-enforced RXRA sumoylation may participate in PML/RARA-driven repression. These data were published in Cancer Cell in 2007. Of note, subsequent work with the EPITRON consortium has demonstrated by CHIP-seq analysis that most, if not all, PML/RARA binding site in APL blasts indeed have RXRA. We then addressed the issue of the importance of cAMP signalling in rexinoid and retinoic response in APL. For this, we generated PML/RARA transgenic mice bearing a point mutation in a serine residue previously demonstrated to be phosphorylated by PKA. To our surprise, this did not alter cAMP sensitivity of the disease, but rather induced a relative retinoic acid (RA) resistance. Investigating the basis for this altered RA-sensitivity, we demonstrated that this RARA or PML/RARA mutant is less susceptible to RA-induced catabolism than the parental proteins, while transcriptional activation remains essentially unchanged. Interestingly, in vivo, short-term differentiation is unaltered, while the loss of the clonogenic potential (and hence disease regression), is dramatically affected. We also discovered that the variant PLZF/RARA-driven APLs perfectly differentiate, but do not loose self-renewal. This has allowed us to propose a renewed model for APL response to RA whereby transcriptional activation controls differentiation, while only full PML/RARA degradation by RA allows APL clearance. Importantly, this models accounts for the effects of arsenic trioxide, which only degrades PML/RARA and induces APL clearance in the absence of major ex vivo differentiation. Moreover, it fully accounts for the synergy between RA and arsenic, not for differentiation, but for LIC clearance, through synergistic PML/RARA degradation. These experiments were published in Nature Medicine in 2008 and have been covered by several highly visible editorials. Finally, in an important paper published in Cancer Cell in 2010, we have unraveled the actual mechanism for arsenic-induced PML/RARA degradation, and hence APL clearance in patients. Briefly, we demonstrated that PML is a redox-sensitive protein that many for disulphide bonds upon oxidation. Arsenic poisons the mitochondria (thus inducing reactive oxygen species (ROS) and also directly binds PML or PML/RARA. This allows the targeting to the nuclear matrix, the reformation of PML nuclear bodies, recruitment of the SUMO-dependent ubiquitin ligase RNF4 and degradation of PML or PML/RARA by the proteasome. Importantly, a point mutant that fails to undergo matrix transfer upon oxidation does not respond to arsenic in primary cultures. Moreover, strong oxidants, such as Paraquat, induce APL regression in vivo, demonstrating that arsenic acts at least in part through generation of ROS. Collectively, these experiments explain the curative action of arsenic on APL. These findings have lead to significant media coverage and to the awarding of several research prizes. These advances in our understanding of APL pathogenesis and basis for treatment response have been summarized in a highly visible review in Nature Reviews Cancer in 2010.

List of selected publications:

Jeanne M, Lallemand-Breitenbach V, Ferhi O, Koken M, Le Bras M, Duffort S, Peres L, Berthier C, Soilihi H, Raught B, de Thé H. PML/RARA oxidation and arsenic binding initiate the antileukemia response of As2O3. Cancer Cell. 2010 Jul 13;18(1):88-98.

Nasr R, Guillemin MC, Ferhi O, Soilihi H, Peres L, Berthier C, Rousselot P, Robledo-Sarmiento M, Lallemand-Breitenbach V, Gourmel B, Vitoux D, Pandolfi PP, Rochette-Egly C, Zhu J, de Thé H. Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation. Nat Med. 2008 Dec;14(12):1333-42. Epub 2008 Nov 23. Erratum in: Nat Med. 2009 Jan;15(1):117.

de Thé H, Chen Z. Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. Nat Rev Cancer. 2010 Nov;10(11):775-83. Epub 2010 Oct 22.

Zhu J, Nasr R, Pérès L, Riaucoux-Lormière F, Honoré N, Berthier C, Kamashev D, Zhou J, Vitoux D, Lavau C, de Thé H. RXR is an essential component of the oncogenic PML/RARA complex in vivo. Cancer Cell. 2007 Jul;12(1):23-35.

1.8 PARTNER 8 - UNIMI:

AIMS OF THE PROJECT The project was conceived i) to set up a novel strategy for the generation of a reporter mouse tool to be applied to epi-drug screening and ii) to provide a set of reporter mice to monitor the transcriptional activity of TRAIL, DR5 receptor and P21. INTRODUCTION Oncology drug development is an error prone process displaying one of the highest attrition rate among the current therapeutic areas. In the last twenty years, most of the antineoplastic drugs entering clinical trials failed due to lack of efficacy and toxicity; this is pointing to the insufficient predictive power of the tools adopted in preclinical drug development for evaluating drug efficacy and toxicity (Sharpless and Depinho, 2006). A common trait of preclinical methodologies applied to the development of new chemical entities is the lack of consideration of the spatio/temporal dimension of drug efficacy and toxicity (Rando et al 2010, Boverhof et al 2011). However, it is well known that the nature and the quality of the responses to a drug depends on factors which changes in time and among different target cells. With this rationale in mind we have proposed a project which combined molecular imaging of reporter of epidrug activity and genetically engineered mice to enable the spatio-temporal semi quantitative analysis of molecular events targeted by epidrugs in living mice. To measure the activity of epidrugs in the whole animal, we have chosen to develop reporter systems of two pathways previously described as mediators of epidrug actions in transformed cells: TRAIL-mediated apoptosis and P21-mediated growth arrest. Indeed, induction of both pathways are associated to the responses of cancer cells to HDAC inhibitors and are typically initiated by a transcriptional induction of TRAIL, DR5 and P21 genes in the tumor cells. THE STRATEGY On the basis of this rationale we have elaborated a knock-in strategy to insert a reporter cassette, suitable for multimodality (bioluminescence, fluorescence and PET) in vivo imaging measurements, into the mouse genomic locus encoding for TRAIL, DR5 and P21 genes, with the idea to substitute the expression of the endogenous gene with that of the reporter cassette. A general prerequisite of this strategy was to keep as much as possible the endogenous regulatory sequences of the targeted gene in order to obtain a reliable reporter mouse. The strategy was expected to generate innovative pharmacological tools (reporter mice) to define epidrug actions in vivo based on the measure of TRAIL, DR5 and P21 expression in the normal and neoplastic tissues of living mice. RESULTS 1. Generation and characterization of the vectors for homologous recombination. 1.1 TRAIL vector The vector was designed in order to modify the TRAIL locus with a reporter cassette allowing to generate a reporter mouse for the multimodality imaging of TRAIL expression. A scheme of the final targeting vector together with the strategy for homologous recombination is reported in Fig. 1. The main features of the vector are: the reporter cassette, the homology regions and the selection markers. A modular assembly of each single component of the vector was carried out and required ten main cloning steps. The best homology regions that could be chosen for homologous recombination unfortunately displayed a high degree of repetitive sequences. The repetitive elements covered up to 68 % of the 5' homologous region (HR5') and 37% of the 3' homologous region (HR3'). Although suboptimal for homologous recombination, no alternative possibilities could be found without changing the overall strategy for the generation of the reporter mouse, than we decided to proceed with this vector. The targeting construct was initially tested for its ability to drive the expression of both reporters by transient transfection experiments in COS and SK-NBE cell lines and subsequently used

for ES cells transfection. After four rounds of electroporation experiments we have selected and screened n. 447 ES clones out of which n. 4 clones displayed homologous recombination in Southern blot and PCR analysis. Two clones gave rise to four viable chimeras, one of which transmitted the recombinant allele to the progenies. The transgenic line was bred with a CRE-expressing mouse to remove the neomycin selection marker present inside the genomic locus and the final reporter mouse line was expanded for characterization. 1.2 P21 vector The concept of multimodality imaging used in the generation of the TRAIL vector was pushed forward in the P21 targeting vector; in particular, the reporter cassette of this construct was conceived to express firefly luciferase and the EGFP/sr39tk fusion protein which together allow for bioluminescence, fluorescence and PET imaging. The scheme of the targeting vector and the strategy for homologous recombination are reported in Fig. 2. The P21 regions chosen for homologous recombination had a better profile compared to TRAIL with a much lower degree of repetitive sequences. The repetitive elements covered up to 17 % of the HR5' and 18% of the HR3'. The targeting construct was initially tested for its ability to drive the expression of both reporters by transient transfection experiments in COS and SK-NBE cell lines and the validated construct was used for the transfection of ES cells. After two rounds of electroporation experiments we have selected and screened more than 400 ES clones out of which we obtained 8 clones displaying the correct recombinant allele in Southern blot and PCR analysis. Two ES clones gave rise to five viable chimeras, one of which transmitted the recombinant allele to the progeny. The transgenic line was bred with a CRE-expressing mouse to remove the neomycin selection marker present inside the genomic locus and we expanded the final reporter mouse line for characterization. 2. Characterization and validation of the TRAIL and P21 reporter mouse lines 2.1 TRAIL reporter mouse characterization As first characterization step, we demonstrated that the two reporters were expressed in the mouse tissues and that the level of luciferase was sufficient for the bioluminescence imaging (BLI) procedure (Figure 2). Indeed, the level of luciferase expression was high enough to be detected for both in vivo and ex vivo imaging analysis. The next validation step for the TRAIL reporter mouse was to demonstrate that the endogenous gene expression paralleled reporter gene expression in the mouse organs. To this purpose, we compared luciferase activity with the level of TRAIL mRNA in several tissues (Fig. 4). The data clearly showed that luciferase activity mirrored the mRNA levels in all tissues analyzed. To further strengthen the data demonstrating co-expression between the reporter cassette and TRAIL, we carried out immunohistochemistry experiments on several tissues. The results of these experiments showed that TRAIL and EGFP expression were coincident in the same cells within the tissues analyzed (Fig. 5). Interestingly the highest expression was found not only in the inflammatory cells, but also in the epithelial cells of several organs including lung, stomach, intestine, skin. Very low expression of both TRAIL and EGFP was demonstrated in organs like liver, brain, skeletal muscle. From these data we concluded that in the TRAIL reporter mouse, the expression of the reporter cassette is widely overlapping with the endogenous TRAIL expression. Next, we addressed the question whether the reporter was able to detect also a transcriptional induction of TRAIL expression following physiological stimuli or upon pharmacological treatments. To this purpose we infected mice with myobia, an agent inducing inflammatory reaction and alopecia, and in these mice we were able to detect a strong induction of luciferase activity (Fig. 6). In the brain of the reporter mice, TRAIL and EGFP/luciferase reporters were basically undetectable in most of the cell types. Inducing brain ischemia in these mice, we found an increased expression of TRAIL and

EGFP/luciferase reporters in perivascular astrocytes; immunohistochemistry was clearly demonstrating that both gene were co-expressed in the same cells (Fig. 6). These results demonstrated that pathological conditions inducing TRAIL expression in selected target cells also induced the reporter expression in the same cells of the TRAIL reporter mouse. TRAIL expression could also be triggered by compounds able to induce acute inflammatory/apoptotic responses to specific target tissues. In particular, we tested the induction of TRAIL and reporter genes after treating the TRAIL reporter mouse with an agent (LPS/D-GALN) inducing acute hepatitis in mice and a well known carcinogen (dimethylbenzantracene, DMBA) inducing also a local inflammatory reaction in the mouse skin (Fig.7). Treatment of the right ear of the reporter mouse with DMBA for 9 days was clearly inducing photon emission, a luciferase induction that was not observed in the left ear treated with vehicle; immunohistochemistry analysis of the tissue showed that of TRAIL and EGFP expression was greatly increase in the epithelium of the DMBA treated ear; furthermore, an important infiltration of EGFP/TRAIL positive inflammatory cells could be detected in the derma below the keratinocytes (Figure 7a). In another set of experiments we have treated the TRAL reporter mice with LPS/D-GALN, a potent inducer of an acute liver inflammatory reaction which end up with apoptosis of hepatic cells and a fulminant hepatitis occurring as early as 6 hours after treatment (Sparwasser et al 1997, Mignon et al 1999): in the liver of treated mice we were able to observe a great induction of luciferase activity which paralleled a caspase-3 activation in the hepatic cells (Figure 7b). Finally we studied TRAIL and reporter expression during neoplastic transformation. We induced mammary tumors through a chemical carcinogenesis protocol (Aldaz et al 1996) based on the administration of medroxyprotgesterone acetate and dimethylbenzanthracene (DMBA). Treatment induced an initial mammary hyperplasia which eventually evolved into a full neoplastic transformed phenotype (12-40 weeks after treatment). TRAIL and reporter expression increased during tumor progression (Fig. 8) particularly in the epithelial compartment of the breast (Fig.8 and Fig. 9). Altogether these experiments validated the TRAIL reporter mouse demonstrating that in this model luciferase and EGFP expression mirrors the expression of the endogenous TRAIL; the expression was found (or even more importantly, not found) coincident in the same tissues and in the same cell types in physiological conditions and upon pathological stimulation of TRAIL expression. 2.2 P21 reporter mouse characterization. As first characterization step of the reporter mouse, we demonstrated that the three reporters were expressed in the mouse tissues and that luciferase could be detected by the bioluminescence imaging (BLI) procedure (Figure 10). The photon emission from the P21 mouse was one order magnitude higher as compared to the TRAIL reporter mouse, thus we needed to set the exposition time in a linearity interval of the curve. The emission was not equally distributed among different organs and also within the same organ; luciferase expression evaluated with the enzymatic assay in protein extracts from the different tissues confirmed the variable level of expression ranging from the 130 RLU of the spleen to the 5.200 RLU of the skin. Next, we verified the ability of the P21 reporter mouse to report physiological fluctuations of P21 expression. Since P21 expression is regulated during the normal physiological proliferative changes occurring in the endometrium of female mice during estrous cycle, we expected to observe such fluctuations also in the luciferase expression. To measure these fluctuations we subjected female mice to a daily imaging session for several consecutive days and, in parallel, to a vaginal smear test to verify the phase of the estrous cycle. The results shown in Figure 11 demonstrated that

light emission from the reproductive area is paralleling the expected modulation of P21 expression i.e. during the proliferative phase the reporter expression is low, while is activated when endometrial cells differentiate during the late estrous/metestrous phases; in diestrous the endometrium is set again for the hormonal response of the proestrus phase and P21 is again turned off. These results demonstrate that in the P21 reporter mouse is possible to follow the physiological dynamic of P21 expression within the same living mouse. As a second validation set experiments we wanted to demonstrate the ability of the reporter to detect the changes in P21 expression occurring in response to DNA damages and P53 activation. To this purpose we treated the ear skin of the P21 reporter mouse with DMBA, a DNA damaging agent, and the second ear with vehicle and we measured the dynamic of luciferase expression by in vivo imaging. We were expecting that DNA damages induced by the DMBA treatment were activating the P53 transcriptional response, which in turn is able to induce P21 expression which in the reporter mouse is detectable as a luminescent emission from the treated ear. Indeed, we observed strong bioluminescence emission in the DMBA-treated ears peaking at 5 days and going back near to baseline in about 10 days (Figure 12). The ear treated with vehicle was not showing any light emission indicating that bioluminescence was specifically induced by the DMBA treatment. The results obtained indicate that in the P21 reporter mouse the reporter cassette is induced by the same physiological and toxicological stimuli inducing the transcription of P21 expression. The biosensor is ubiquitously expressed and the bioluminescent response is highly reproducible and with a large dynamic range; these features suggest that the reporter mouse can be used for the profiling of any compound which is supposed to induce the transcription of P21 (Vantaggiato et al). 3. Validation of the reporter mouse technology for the screening of HDAC inhibitors 3.1 TRAIL reporter mouse validation for HDAC inhibitor screening To evaluate whether the reporter mouse technology could be adopted for the screening of HDAC inhibitors we decided to treat the TRAIL reporter mice with SAHA (Vorinostat), a well known compound of this class. Since HDAC inhibitors were shown to selectively kill tumor cells through the induction of TRAIL expression (Nebbioso et al 2005, Insinga et al 2005), we hypothesized that it is possible to observe increased level of TRAIL only in transformed cells; alternatively, it is also possible that TRAIL can be induced in all cells, but only tumor cells are able to initiate an apoptotic programme. To test these hypotheses, we treated with SAHA, TRAIL reporter mice carrying or not carrying mammary neoplasia. The results of these experiments showed that SAHA is not able to induce the reporter expression in normal tissues (Fig.13), while induced TRAIL expression in the neoplastic breast of the TRAIL reporter mouse (Fig.14). These experiments showed that SAHA is able to induce TRAIL expression selectively in the neoplastic tissue. From these data we can conclude that we have generated and validated the TRAIL reporter mouse which can efficiently report the ability of HDAC inhibitors to induce TRAIL expression selectively in tumor tissues (Vantaggiato et al). The model can be applied to study in vivo candidate drugs supposed to induce TRAIL expression and at the same time to evaluate the selectively of these compounds to tumor tissue to avoid potentially relevant side effects linked to ectopical activation of this pathway in normal tissues. 3.2 Intracellular nuclear receptor reporter mice for HDAC inhibitor screening Preliminary data in our lab showed that ER and PPAR reporter systems were able also to measure the activity of HDAC inhibitors. Indeed, both transient and stably transfected cells with ERE-Luc or PPRE-Luc reporter systems were responding to HDAC inhibition with an increased production of luciferase. Initially, we addressed the

question whether the observed induction is due to a specific increase in nuclear receptor transcriptional activity. We demonstrated that in MCF-7 cells stably transfected with an ERE-Luc reporter treatment with TSA, DAC60 and MC1568 were able to stimulate ERE-mediated transcription and that this increase was inhibited by the ER pure antagonist ICI 182,780 (Fig.15). Therefore we concluded that the ERE-Luc and PPRE-Luc reporter systems might constitute an indirect way to measure the activity of HDAC inhibitors. Our laboratory generated and validated ER (Ciana et al 2003) and PPAR (Biserni et al 2008) reporter mice, transgenic mice where the reporter system can be ubiquitously activated in the presence of an activated intracellular receptor. Since we demonstrated in cells that HDAC inhibitors can regulate transcription from these reporter systems, we have postulated the possibility to use these reporter mice to profile the activity of HDAC inhibitors in vivo. To test this hypothesis, we treated s.c. ERE-Luc reporter mice with TSA 0.5 mg/Kg daily for three days and the last day we performed an in vivo imaging analysis of luciferase expression (Fig. 16). The results were quite encouraging showing that ERE-Luc reporter system could be induced in several body area of the mouse; in particular, in the area corresponding to the liver, we observed an interesting induction of the reporter gene expression. Next, we repeated the experiment with different group of animal treating them with 0.5 mg/Kg TSA or vehicle for three days; for the last treatment we administered increasing doses of TSA (0, 0.05, 0.5, 5 mg/Kg) (Figure 17). In vivo imaging revealed an interesting dose dependent activation of the photon emission in parallel with increased luciferase expression in specific, but not all target tissues. Indeed we observed a dose dependent luciferase induction in liver, prostate and bone, while we did not observed any effect in testis and thymus. As demonstrated by several studies from our other laboratories the ERE-Luc biosensor is ubiquitously active in our mice, thus, we have observed a specific profile of HDAC inhibition in vivo induced by TSA that might highlight a tissue specific activity of this drug previously unrecognized. Collectively, our in vitro and in vivo data points to the use of the ERE-Luc reporter mouse as tools to investigate the pharmacological activity of these compounds. DEVIATIONS FROM THE ORIGINAL PLAN We have generated and validated two reporter mice out of the three planned in the beginning of the project. The reason was strategic: we learnt that TRAIL and P21 reporter mice were optimal tools to measure the efficacy of a potential anti-tumor activity of HDAC inhibitors (rising of TRAIL and P21 expression); however, this activity can manifest only in neoplastic tissues (see data reported in 3.1, Fig.12-13), while in normal tissue a rising in TRAIL or P21 expression is not expected following HDAC inhibitor treatment. Therefore the reporter mice generated are valuable to study efficacy in neoplastic tissue and to highlight sites of potential toxicity in normal tissues (sites were TRAIL or P21 are enhanced with a potential impact in the normal tissue homeostasis). However, TRAIL and P21 reporter mice do not provide information about the profile of target activity in vivo; the generation of a DR4/5 reporter mouse would have not helped to this purpose. For these reasons we decided to abandon the generation of the DR4/5 reporter mouse and to undertake a different strategy for the realization of a tool which is allowing the pharmacological profiling of HDAC inhibitors activity in the mouse. Thus, we have developed and validated intracellular receptor reporter cells and mice for the screening of epidrugs: this is a novel concept which might help in the definition of epidrugs activity in normal tissues, a piece of information which complement the data that can be obtained with the TRAIl and P21 reporter mice. Furthermore, we extensively characterized and validated the reporter mouse model (especially the TRAIL reporter mouse) far beyond what was originally planned. SCIENTIFIC

IMPACT With this project we have provided the scientific community with unprecedented and extraordinarily powerful tools for studying apoptosis, innate immunity and growth arrest in living mice. Impact to basic research Reporter mice allow to study molecular events in the temporal and spatial (all body sites) dimension (Maggi and Ciana, 2005). The importance of studying molecular events in the spatio-temporal dimension is now well recognized by the scientific community. This is especially true for neoplastic transformation, which is a process characterized by a series of genetic and epigenetic mutations that evolve over years even decades; paradoxically most methods developed to study these changes are static and do not provide a dynamic view of the molecular process involved in cancer transformation (Stell et al 2007, Ramachandran et al 2011). The impact of this type of information in the study of cancer in cannot be predicted, but preliminary evidence already produced in the lab using the tools we generated in the frame of the EPITRON project, already showed that we can look at the tumorigenesis process from a novel point of view. A point of view that allows: i) a novel way to staging the transforming tissue based on the molecular changing occurring in time during tumor progression and ii) a novel way to look into the participation of other tissues (predominantly, but not restricted to immune system) to the transforming event. We believe introducing the spatio/temporal information into the cancer investigation will allow a better understanding of the mechanicistical acquisition of the traits characterizing cancer cell development in the body. Impact to antineoplastic drug discovery The impact of reporter mice to pharmacological studies in general (Maggi et al 2004, Maggi and Ciana 2005) and to epidrug discovery in particular, can be important for addressing two highly debated themes in the field: i) the need of an innovative definition of drug efficacy and ii) a novel way to investigate drug toxicity. Indeed, lack of efficacy and undesired toxicity are the main causes of drug withdrawal in the late stages of drug discovery, a harsh problem involving 95% of drugs entering in clinical trials. This is highlighting the insufficient information provided by current preclinical methods used to predict efficacy and toxicity. These tools do not provide any information concerning the dynamic action of drugs in the body. The definition of drug efficacy and toxicity is depending on the target organ (space dimension, where in the body the drug is active) and on the time when measurements are taken (time dimension). Reporter mice allow to follow drug action in space and time thus are candidate tools to better define drug action (Rando et al 2010, Della Torre et al 2011). With the EPITRON project we have generated novel type of biosensors that were inserted in transgenic mice creating a model for the spatio/temporal definition of epidrug efficacy and toxicity (Vantaggiato et al 2011). We have proven that the tools generated can be useful to define in living mice: i) which normal and neoplastic tissues are targeted by the drug, ii) which is the potency of HDAC inhibition, iii) which is the efficacy in inducing anti-cancer signals and iv) which are the normal tissues in which there is an ectopical inhibition of anti-cancer signals. Integrating these information in the time dimension, will allow a better definition of the epidrug efficacy and toxicity. The tools generated in EPITRON will certainly constitute a proof-of-principle of a novel approach to the preclinical definition of drug action, which, we believe, will be extended also outside the epidrug field to other class of anti-neoplastic drugs.

REFERENCES

-Sharpless NE, Depinho RA. The mighty mouse: genetically engineered mouse models in cancer drug development. Nat Rev Drug Discov. 2006 Sep;5(9):741-54.

- Rando G, Horner D, Biserni A, Ramachandran B, Caruso D, Ciana P, Komm B, Maggi A. An innovative method to classify SERMs based on the dynamics of estrogen receptor transcriptional activity in living animals. Mol Endocrinol. 2010 Apr;24(4):735-44.

- Boverhof DR, Chamberlain MP, Elcombe CR, Gonzalez FJ, Heflich RH, Hernández LG, Jacobs AC, Jacobson Kram D, Luijten M, Maggi A, Manjanatha MG, Benthem J, Gollapudi BB. Transgenic animal models in toxicology: historical perspectives and future outlook. Toxicol Sci. 2011 Jun;121(2):207-33.

- Dondi D, Piccolella M, Biserni A, Della Torre S, Ramachandran B, Locatelli A, Rusmini P, Sau D, Caruso D, Maggi A, Ciana P, Poletti A. Estrogen receptor beta and the progression of prostate cancer: role of 5alpha-androstane-3beta,17beta-diol. Endocr Relat Cancer. 2010 Jul 28;17(3):731-42.

- Sparwasser T, Miethke T, Lipford G, Erdmann A, Häcker H, Heeg K, Wagner H. Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-alpha-mediated shock. Eur J Immunol. 1997 Jul;27(7):1671-9.

- Mignon A, Rouquet N, Fabre M, Martin S, Pagès JC, Dhainaut JF, Kahn A, Briand P, Joulin V. LPS challenge in D-galactosamine-sensitized mice accounts for caspase-dependent fulminant hepatitis, not for septic shock. Am J Respir Crit Care Med. 1999 Apr;159(4 Pt 1):1308-15.

- Aldaz CM, Liao QY, LaBate M, Johnston DA. Medroxyprogesterone acetate accelerates the development and increases the incidence of mouse mammary tumors induced by dimethylbenzanthracene. Carcinogenesis. 1996 Sep;17(9):2069-72.

- Insinga A, Monestiroli S, Ronzoni S, Gelmetti V, Marchesi F, Viale A, Altucci L, Nervi C, Minucci S, Pelicci PG. Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat Med. 2005 Jan;11(1):71-6.

- Nebbioso A, Clarke N, Voltz E, Germain E, Ambrosino C, Bontempo P, Alvarez R, Schiavone EM, Ferrara F, Bresciani F, Weisz A, de Lera AR, Gronemeyer H, Altucci L. Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. Nat Med. 2005 Jan;11(1):77-84.

- Vantaggiato C, Maravigna L, Maggi A. and Ciana P. Imaging of trail expression in reporter mice. Manuscript in preparation (a)

- Vantaggiato C, Maravigna L, Maggi A. and Ciana P. Imaging of growth arrest in reporter mice. Manuscript in preparation (b)

- Ciana P, Raviscioni M, Mussi P, Vegeto E, Que I, Parker MG, Lowik C, Maggi A. In vivo imaging of transcriptionally active estrogen receptors. Nat Med. 2003 Jan;9(1):82-6.

- Biserni A, Giannessi F, Sciarroni AF, Milazzo FM, Maggi A, Ciana P. In vivo imaging reveals selective peroxisome proliferator activated receptor modulator activity of the synthetic ligand 3-(1-(4-chlorobenzyl)-3-t-butylthio-5-isopropylindol-2-yl)-2,2-dimethylpropanoic acid (MK-886). Mol Pharmacol. 2008 May;73(5):1434-43.

- Braun RE, Lo D, Pinkert CA, Widera G, Flavell RA, Palmiter RD, Brinster RL. Infertility in male transgenic mice: disruption of sperm development by HSV-tk expression in postmeiotic germ cells. Biol Reprod. 1990 Oct;43(4):684-93.

- Maggi A, Ciana P. Reporter mice and drug discovery and development. Nat Rev Drug Discov. 2005 Mar;4(3):249-55.

- Ramachandran B, Stell A, Maravigna L, Maggi A, Ciana P. Novel insights on imaging sex hormone-dependent tumourigenesis in vivo. Endocr Relat Cancer. 2011 Apr 2;18(3):R41-51.

- Stell A, Biserni A, Della Torre S, Rando G, Ramachandran B, Ottobrini L, Lucignani G, Maggi A, Ciana P. Cancer modeling: modern imaging applications in the generation of novel animal model systems to study cancer progression and therapy. Int J Biochem Cell Biol. 2007;39(7-8):1288-96.

- Maggi A, Ottobrini L, Biserni A, Lucignani G, Ciana P. Techniques: reporter mice - a new way to look at drug action. Trends Pharmacol Sci. 2004 Jun;25(6):337-42 - Della Torre S, Biserni A, Rando G, Monteleone G, Ciana P, Komm B, Maggi A. The conundrum of estrogen receptor oscillatory activity in the search for an appropriate hormone replacement therapy. Endocrinology. 2011 Jun;152(6):2256-65.

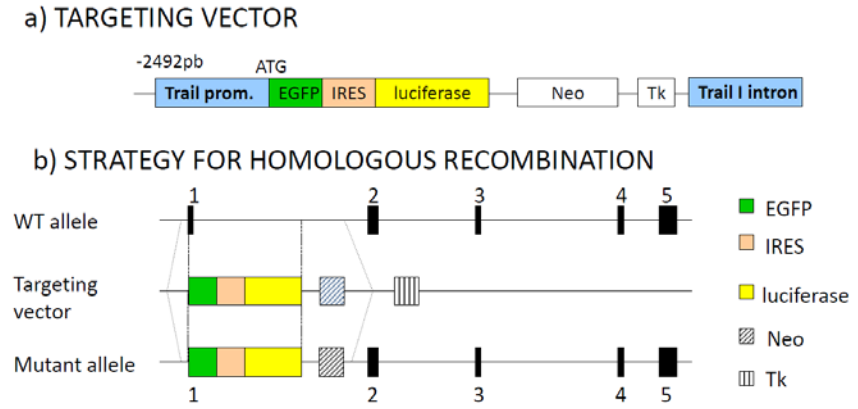


Figure 1. a) Features of the targeting vector: i) the regions for the homologous recombination were amplified from the DNA of Sv129 mouse strain and subcloned into a cloning vector; they consists in 2.5 Kb sequences upstream the TRAIL promoter and 4.5 Kb intron 1 sequences; ii) the reporter cassette contains EGFP and firefly luciferase reporter genes transcribed in a bicistronic mRNA: for the EGFP, care was taken to preserve the Kozak and ATG from the endogenous TRAIL, while the expression of luciferase (as a second gene in the bicstronic mRNA) was optimized trying different combination Kozak surrounding the ATG sequences; iii) neo and tk markers for the selection of ES cells were obtained from the targeting vector pFM54. b) Schematic representation of the homologous recombination reaction expected in mouse ES cells.

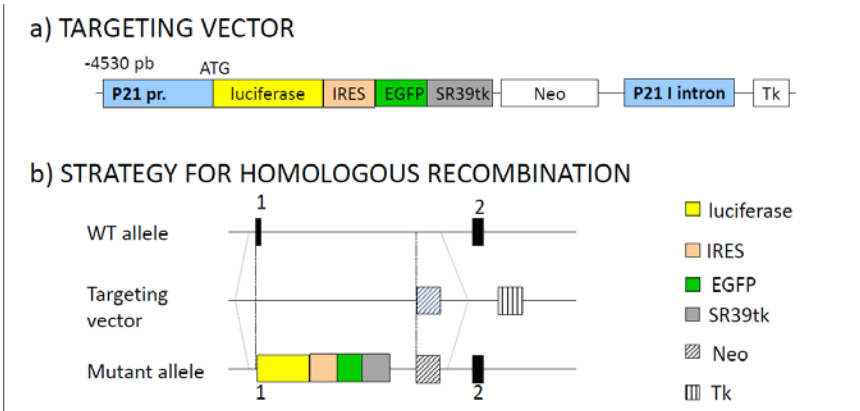


Figure 2. a) Features of the targeting vector: i) the regions for the homologous recombination were amplified from the DNA of Sv129 mouse strain and subcloned into a cloning vector; they consists in 4.5 Kb sequences spanning the upstream regions of the P21 promoter and 2.5 Kb sequences spanning the intron 1 regions; ii) the reporter cassette contains the firefly luciferase and for the EGFP/sr39tk fusion protein which are transcribed in a bicistronic mRNA: for the luciferase, the Kozak sequence and ATG from the endogenous P21 were conserved in the construction, while for the EGFP/sr39tk the expression was optimized trying different combination of sequences surrounding the ATG; iii) neo and tk cassettes were from the targeting vector pFM54. b) Schematic representation of the homologous recombination reaction expected in mouse ES cells.

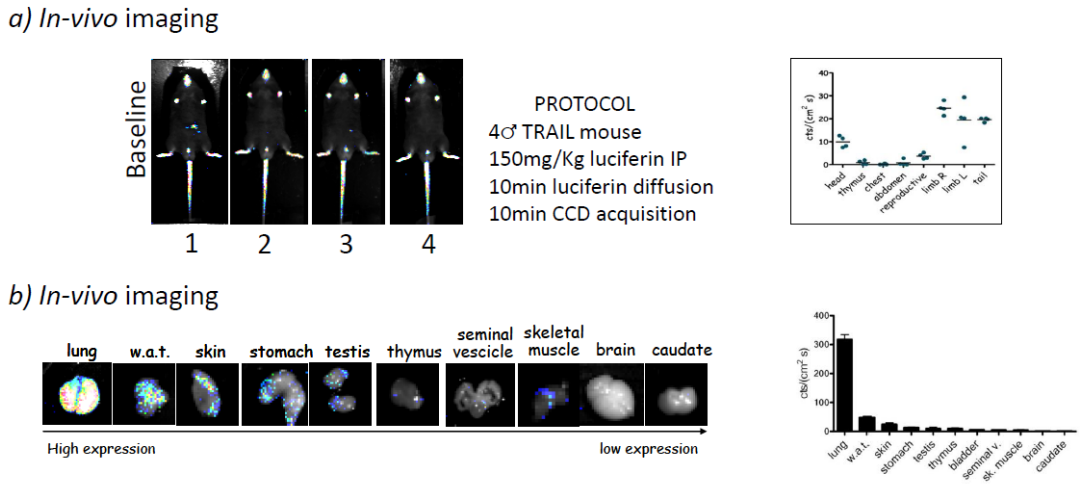


Fig. 3. a) pictures represent a non-invasive bioluminescence imaging (Dondi et al 2010) experiment carried out with four individuals of the TRAIL reporter mouse. Mice received an i.p. injection of 50 mg/kg D-luciferin (Promega, Madison, WI) 20 min before bioluminescence quantification, to achieve a uniform biodistribution of the substrate; imaging was carried out with a Night Owl imaging unit (Berthold Technologies, Bad Wildbad, Germany) consisting of a Peltier-cooled charge-coupled device slowscan camera equipped with a 25-mm, f/0.95 lens. Pictures were generated by a Night Owl LB981 image processor and transferred via video cable to a peripheral component interconnect frame grabber using WinLight32 software (Berthold Technologies). b) *Ex vivo* imaging of the explanted tissues showing the distribution of TRAIL expression (photon emission); graph represents the quantification of photon emission from the explanted tissues of the mouse. Photon emission was integrated over a period of 5 min; light emission is expressed as number of counts/cm^2 per second.

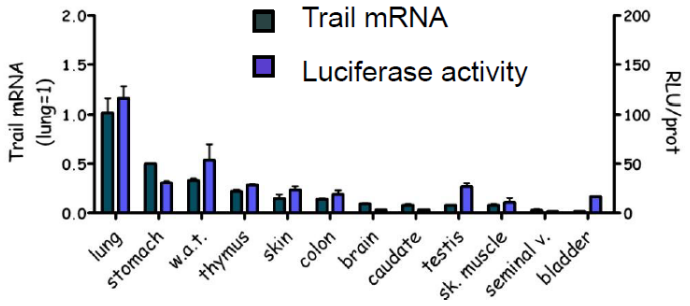


Figure 4. Real time PCR was carried out to measure the TRAIL mRNA in several organs of four males of the TRAIL reporter mouse line. In the same tissues luciferase amount was evaluated using an enzymatic assay on protein extracts.

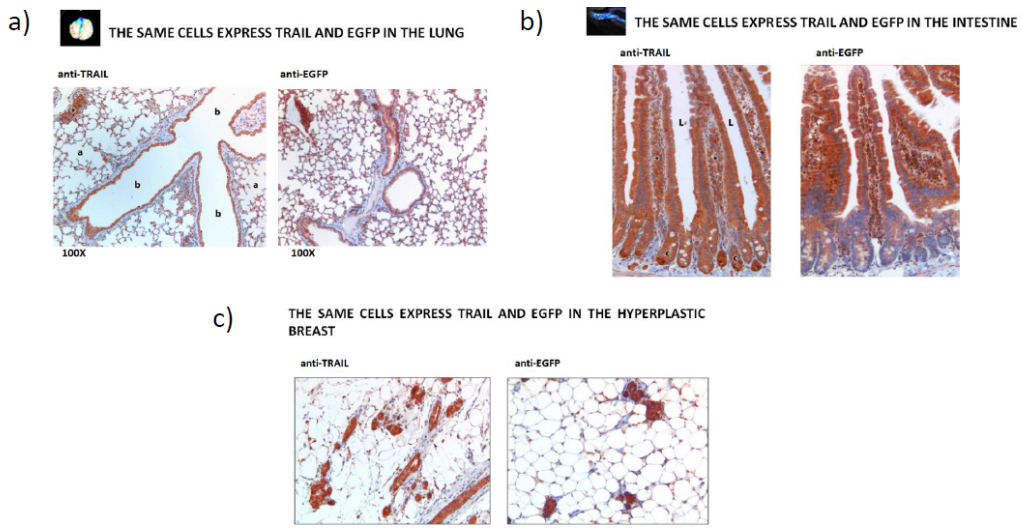


Figure 5 Immunohistochemistry analysis of EGFP and TRAIL expression in lung, intestine and breast tissues. a) Immunohistochemistry in the lung showed high TRAIL and EGFP expression in the bronchiole (b) and in the infiltrating macrophages, medium/low expression in the alveolar (a) epithelium, while no signal could be detected in the myoblast/stromal component. b) EGFP and TRAIL expression in the intestine wasfound high in the lumen (L) epithelium and in the inflammatory cells (*) present inside the villi, while no expression was detectable in the myoblast/stromal component. c) In the hyperplastic breast (induced by dimethylbenzantracene treatment) EGFP and TRAIL were both expressed in the duct and lobule epithelial cells while both genes were not detectable in the myoblast/stromal component.

a) Pathogen-induced inflammation induces bioluminescence in the skin of TRAIL reporter mice

b) TRAIL and EGFP induction in perivascular astrocytes during brain ischemia

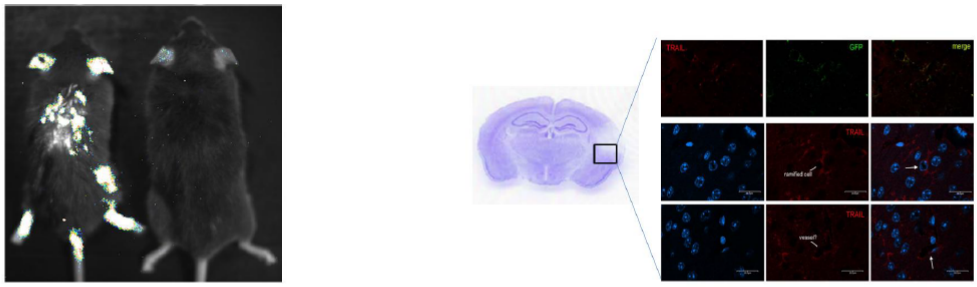
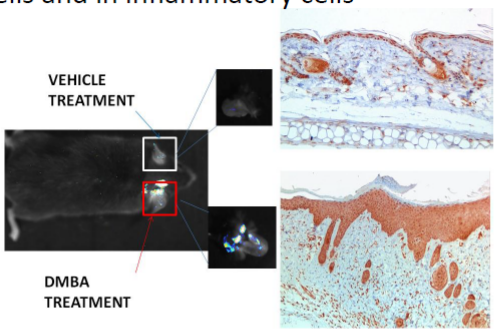


Figure 6. a) BLI of TRAIL reporter mice infected by myobia agent a parasite mite causing alopecia (left) or unaffected (right). b) Perivascular astrocytes are co-espressing TRAIL and EGFP in the ischemic brain as revealed by immunohistochemistry analysis.

a) DMBA induces TRAIL in skin epithelial cells and in inflammatory cells

b) LPS/D-GalN induces luciferase and apoptosis in the liver of TRAIL reporter mice



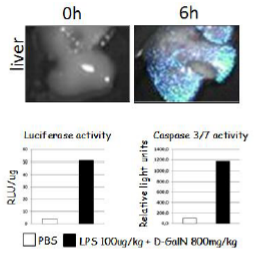


Figure 7. a) BLI was carried out 9 days after a single streak of DMBA or vehicle respectively on the right or left ear of the mouse. Light emission is correlating with a strong increase expression of the TRAIL gene in the skin epithelium and in the infiltrating inflammatory cells. b) Ex vivo imaging of the liver explanted from two TRAIL reporter mice treated i.p. with vehicle or LPS (100ug/Kg) + D-GalN (800 mg/kg).

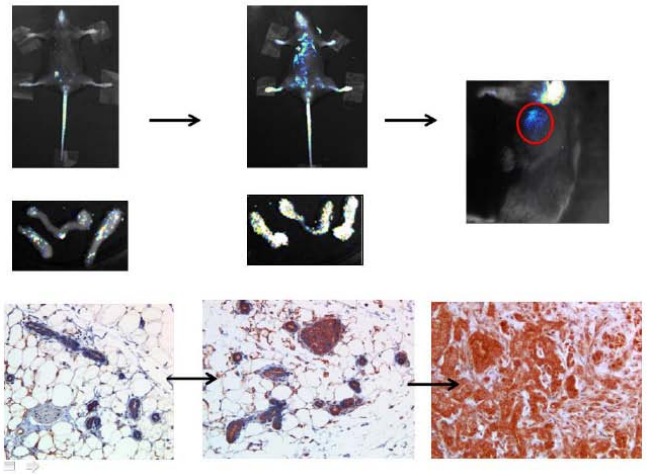


Figure 8 *In vivo*, *ex vivo* imaging and immunohistochemical analysis of photon emission and TRAIL expression during breast cancer progression. Immunohistochemistry analysis was performed using anti-TRAIL antibody.

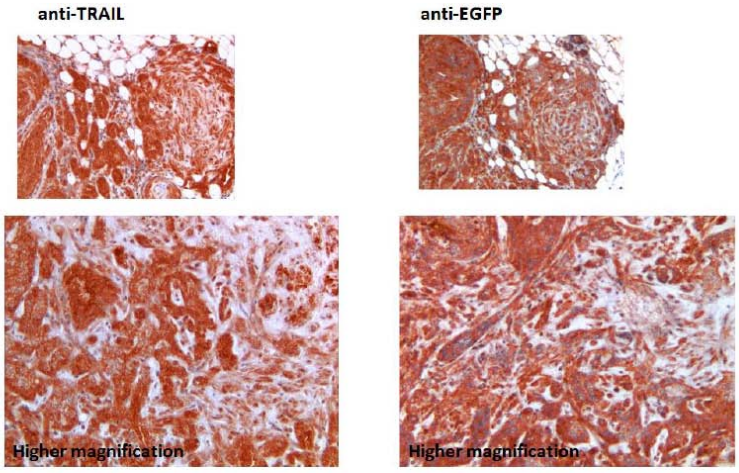


Figure 9. Immunohistochemistry analysis of TRAIL and EGFP expression in mammary adenocarcinoma induced by MPA/DMBA treatment in the TRAIL reporter mice.

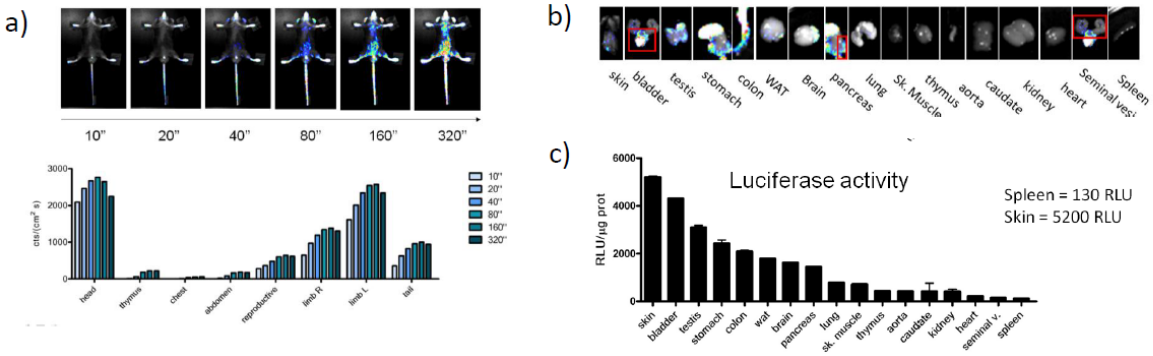


Figure 10. a) BLI analysis of male P21 reporter mouse I.P injected with 25 mg/Kg luciferin and visualized for the indicated times. Photon emission was quantified within the indicated body areas. b) P21 reporter mouse *ex vivo* imaging analysis demonstrated an uneven distribution of luciferase in the mouse tissues. C) Basal luciferase activity measured by enzymatic assay on the protein extracts obtained from the indicated tissues.

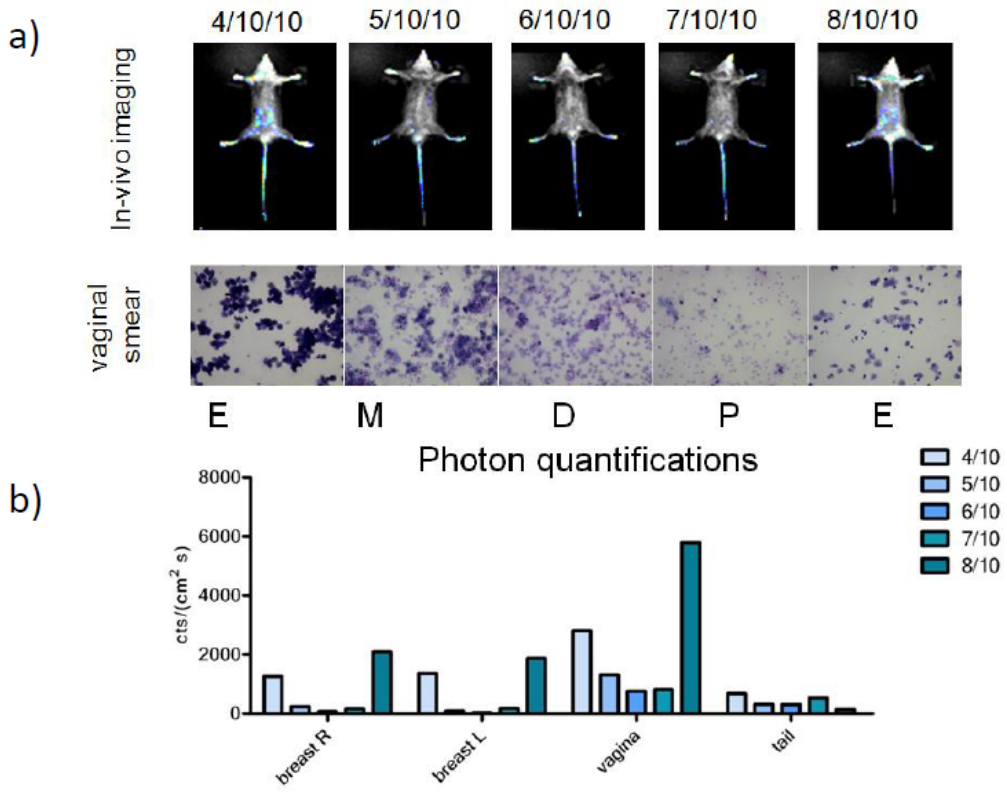


Figure 11. BLI analysis of P21 expression during the estrous cycle. A) Representative female mouse of the P21 line subjected to daily BLI and vaginal smear analysis. B) Quantification of the photons emitted from the indicated area of the animal.

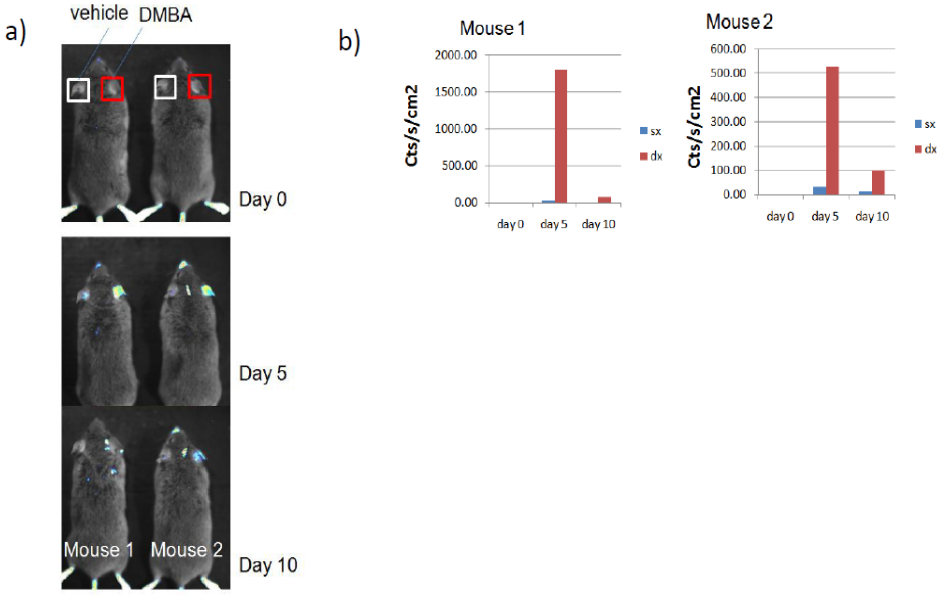


Figure 12. Time-course analysis of luciferase expression in the ear skin of the P21 reporter mouse after DMBA treatment. a) BLI analysis of luciferase expression after 0, 5 and 10 days. b) Graphs represent the quantification of photon emission of the area showed in the picture (white area = vehicle treated ear, red area = DMBA treated ear.

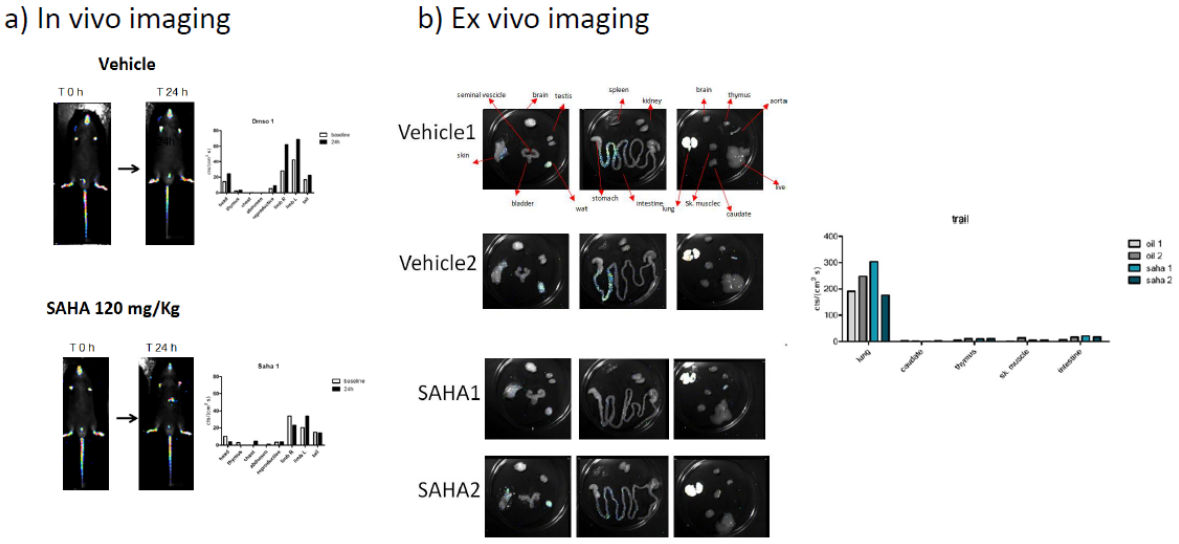


Figure 13. a) Pictures are representative of the in vivo imaging analysis carried out on normal TRAIL reporter mice treated with vehicle or SAHA b) ex-vivo imaging analysis on the organs explanted from the mice treated as in a).

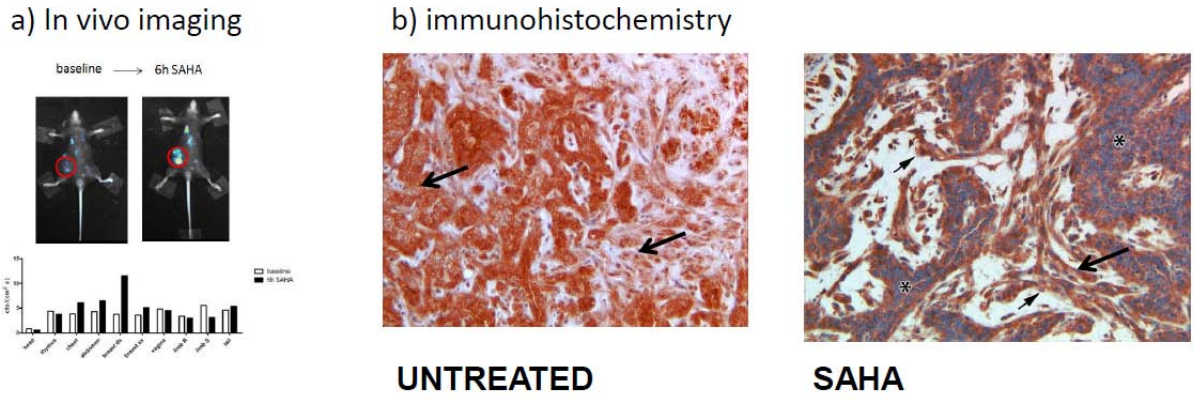


Figure 14. In vivo imaging of the treatment of a TRAIL reporter mouse carrying a mammary tumor treated with 120 mg/Kg SAHA. Photon emission was clearly visible in the tumor area. Immunohistochemistry showed the effects of SAHA treatment at the cellular levels. Arrows indicate tumor cell component (myoblast) not expressing TRAIL becoming positively stained after treatment.

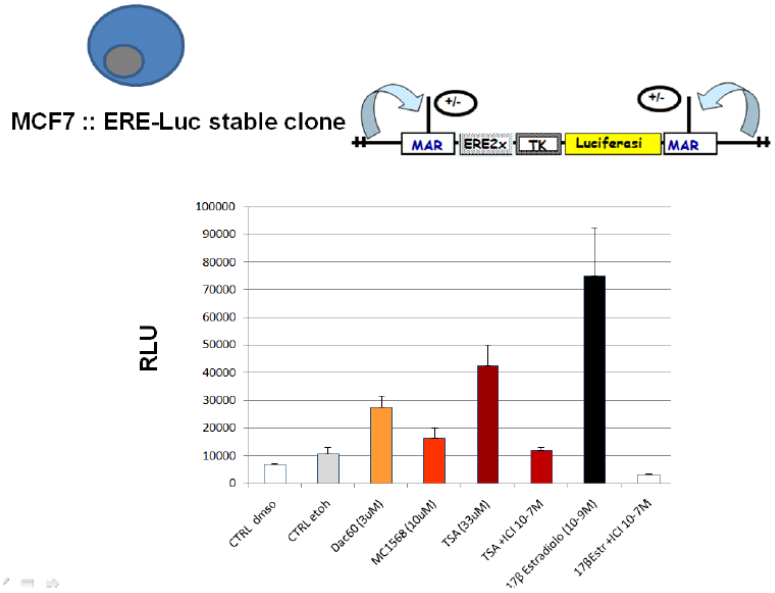


Figure 15. Effect of the HDACi on the estrogen receptor reporter system (ERE-Luc) in MCF-7 stably transfected clones. Several HDAC inhibitors (DAC60, MC1568. TSA) were proven able to indude ERE-Luc reporter system. Cells were treated with the indicated compounds for 24 hours.

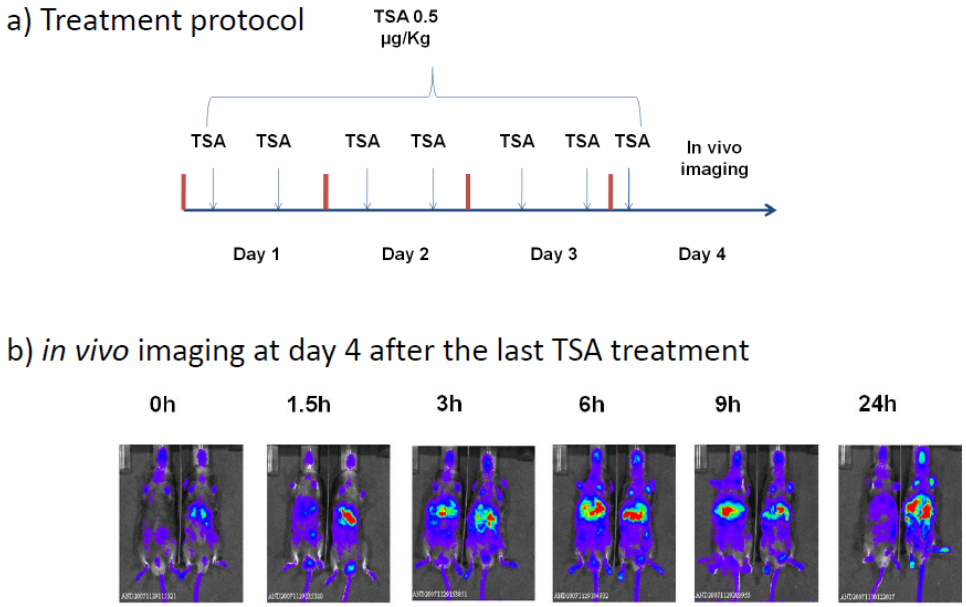


Figure 16. a) protocol for the treatment of ERE-Luc mice with TSA b) time course of photon emission

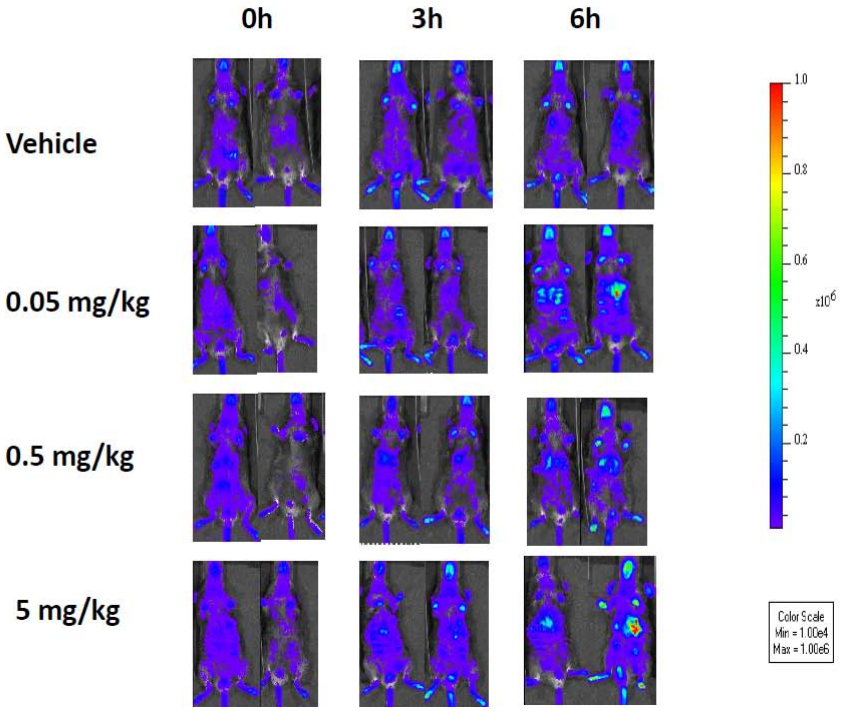


Figure 17. In vivo imaging of ER activation after TSA treatment of the ERE-Luc reporter mice with increasing dosis of the drug.

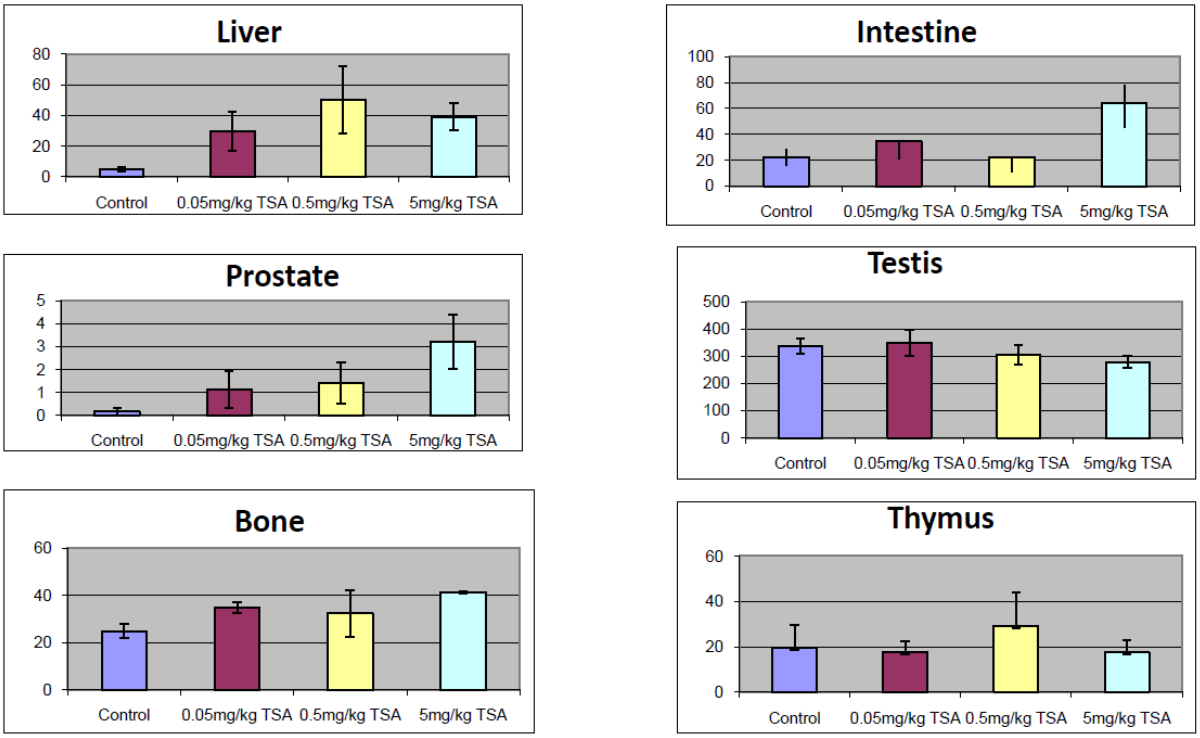


Figure 18. Ex vivo analysis of luciferase induction in the tissues of the ERE-Luc mice after increasing doses of TSA.

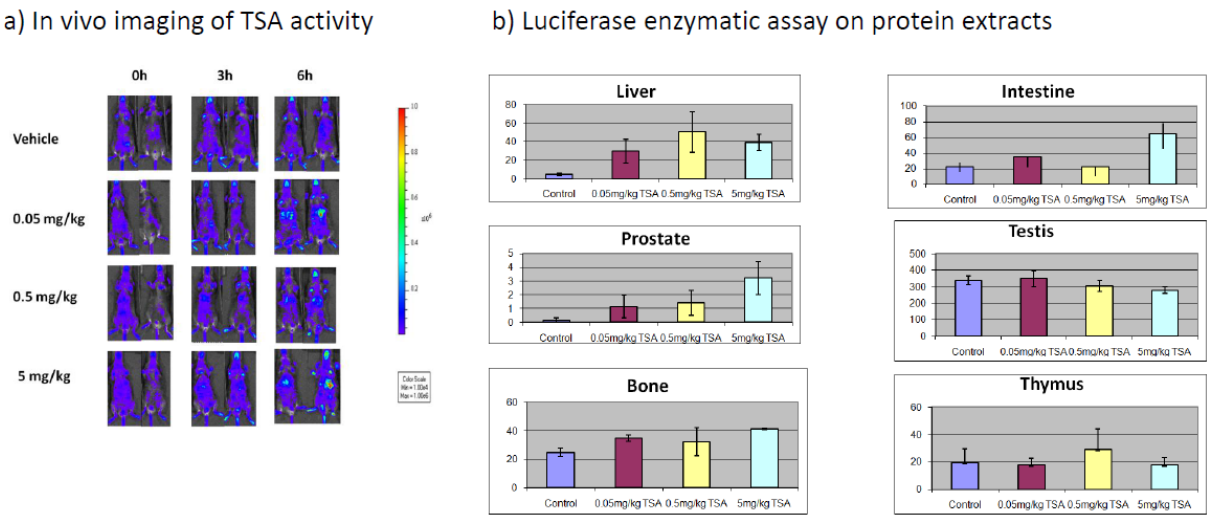


Figure 19. In vivo imaging of the TSA activity in the ERE-Luc reporter mice a) Photon emission is increased in a dose dependent manner b) Luciferase activity measured in tissue extracts reveal dose-dependent activity of TSA in liver, prostate, bone but not in testis or thymus.

1.9 PARTNER 9 - UCAM/WCRUK:

1. A chromodomain switch mediated by histone H3 Lys 4 acetylation regulates heterochromatin assembly
   Blerta Xhemalce and Tony Kouzarides1 Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge CB2 1QN, United Kingdom

   Chromodomain proteins (Chp1/Chp2/Swi6/Clr4) bind to methylated H3K9 (H3K9me) and regulate pericentric heterochromatin in fission yeast. Chp1 and Clr4 (H3K9-HMT), bind transcriptionally active heterochromatin, whereas Chp2/Swi6 (HP1 homologs) are recruited during the inactive state. We show that H3K4 acetylation (H3K4ac) plays a role in the transition of dimethylated H3K9 (H3K9me2) occupancy from Chp1/Clr4 to Chp2/Swi6. H3K4ac, mediated by Mst1, is enriched at pericentromeres concomitantly with heterochromatin reassembly. H3K4R (Lys / Arg) mutation increases Chp1 and decreases Chp2/Swi6 pericentric occupancy and exhibits centromeric desilencing. Consistent with structural data, H3K4ac specifically reduces Chp1/Clr4 affinity to H3K9me. We propose that H3K4ac mediates a chromodomain switch from Chp1/Clr4 to Swi6/Chp2 to allow heterochromatin reassembly. Supplemental material is available at http://www.genesdev.org. Received November 3, 2009; revised version accepted February 12, 2010.

2. Arginine methylation at histone H3R2 controls deposition of H3K4 trimethylation
   Antonis Kirmizis1, Helena Santos-Rosa1, Christopher J. Penkett2, Michael A. Singer3, Michiel Vermeulen4, Matthias Mann4, Ju¨rg Ba¨hler2, Roland D. Green3 & Tony Kouzarides1

   Modifications on histones control important biological processes through their effects on chromatin structure1–3. Methylation at lysine 4 on histone H3 (H3K4) is found at the 59 end of active genes and contributes to transcriptional activation by recruiting chromatin-remodelling enzymes4,5. An adjacent arginine residue (H3R2) is also known to be asymmetrically dimethylated (H3R2me2a) in mammalian cells6, but its location within genes and its function in transcription are unknown. Here we show that H3R2 is also methylated in budding yeast (Saccharomyces cerevisiae), and by using an antibody specific for H3R2me2a in a chromatin immunoprecipitation-on-chip analysis we determine the distribution of this modification on the entire yeast genome. We find that H3R2me2a is enriched throughout all heterochromatic loci and inactive euchromatic genes and is present at the 39 end of moderately transcribed genes. In all cases the pattern of H3R2 methylation is mutually exclusive with the trimethyl form of H3K4 (H3K4me3). We show that methylation at H3R2 abrogates the trimethylation of H3K4 by the Set1 methyltransferase. The specific effect on H3K4me3 results from the occlusion of Spp1, a Set1 methyltransferase subunit necessary for trimethylation. Thus, the inability of Spp1 to recognize H3 methylated at R2 prevents Set1 from trimethylating H3K4. These results provide the first mechanistic

3. JAK2 phosphorylates histone H3Y41 and excludes HP1a from chromatin

Mark A. Dawson1,2*, Andrew J. Bannister3*, Berthold Go¨ttgens1, Samuel D. Foster1, Till Bartke3, Anthony R. Green1,2* & Tony Kouzarides

Activation of Janus kinase 2 (JAK2) by chromosomal translocations or point mutations is a frequent event in haematological malignancies1–6. JAK2 is a non-receptor tyrosine kinase that regulates several cellular processes by inducing cytoplasmic signalling cascades. Here we show that human JAK2 is present in the nucleus of haematopoietic cells and directly phosphorylates Tyr 41 (Y41) on histone H3. Heterochromatin protein 1a (HP1a), but not HP1b, specifically binds to this region of H3 through its chromo-shadow domain. Phosphorylation of H3Y41 by JAK2 prevents this binding. Inhibition of JAK2 activity in human leukaemic cells decreases both the expression of the haematopoietic oncogene lmo2 and the phosphorylation of H3Y41 at its promoter, while simultaneously increasing the binding of HP1a at the same site.These results identify a previously unrecognized nuclear role for JAK2 in the phosphorylation of H3Y41 and reveal a direct mechanistic link between two genes, jak2 and lmo2, involved in normal haematopoiesis and leukaemia1–9.

4. Distinct transcriptional outputs associated with mono- and dimethylated histone H3 arginine 2
   Antonis Kirmizis1, Helena Santos-Rosa1, Christopher J Penkett2, Michael A Singer3, Roland D Green3 & Tony Kouzarides1
   Dimethylation of histone H3 Arg2 (H3R2me2) maintains transcriptional silencing by inhibiting Set1 mediated trimethylation of H3K4. Here we demonstrate that Arg2 is also monomethylated (H3R2me1) in yeast but that its functional characteristics are distinct from H3R2me2: (i) H3R2me1 does not inhibit histone H3 Lys4 (H3K4) methylation; (ii) it is present throughout the coding region of genes; and (iii) it correlates with active transcription. Collectively, these results indicate that different H3R2 methylation states have defined roles in gene expression.

5. Nucleosome-Interacting Proteins Regulated by DNA and Histone Methylation
   Till Bartke,1 Michiel Vermeulen,2,3 Blerta Xhemalce,1 Samuel C. Robson,1 Matthias Mann,2 and Tony Kouzarides1,* 1The Gurdon Institute and Department of Pathology, Tennis Court Road, Cambridge CB2 1QN, UK 2Department of Proteomics and Signal Transduction, Max-Planck-Institute for Biochemistry, D-82152 Martinsried, Germany 3Present address: Department of Physiological Chemistry and Cancer Genomics Centre, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands *Correspondence: t.kouzarides@gurdon.cam.ac.uk

   SUMMARY Modifications on histones or on DNA recruit proteins that regulate chromatin function. Here, we use nucleosomes methylated on DNA and on histone H3 in an affinity assay, in conjunction with a SILAC-based proteomic analysis, to identify ‘‘crosstalk’’ between these two distinct classes of modification. Our analysis reveals proteins whose binding to nucleosomes is regulated by methylation of CpGs, H3K4, H3K9, and H3K27 or a combination thereof. We identify the origin recognition complex (ORC), including LRWD1 as a subunit, to be a methylation-sensitive nucleosome interactor that is recruited cooperatively by DNA and histone

methylation. Other interactors, such as the lysine demethylase Fbxl11/KDM2A, recognize nucleosomes methylated on histones, but their recruitment is disrupted by DNA methylation. These data establish SILAC nucleosome affinity purifications (SNAP) as a tool for studying the dynamics between different chromatin modifications and provide a modification binding ‘‘profile’’ for proteins regulated by DNA and histone methylation.

List of selected publications:

Bartke T, Vermeulen M, Xhemalce B, Robson SC, Mann M, Kouzarides T. Nucleosome-interacting proteins regulated by DNA and histone methylation. Cell. 2010 Oct 29;143(3):470-84

Kirmizis A, Santos-Rosa H, Penkett CJ, Singer MA, Green RD, Kouzarides T. Distinct transcriptional outputs associated with mono- and dimethylated histone H3 arginine 2. Nat Struct Mol Biol. 2009 Apr;16(4):449-51. Epub 2009 Mar 8.

Xhemalce B, Kouzarides T. A chromodomain switch mediated by histone H3 Lys 4 acetylation regulates heterochromatin assembly. Genes Dev. 2010 Apr 1;24(7):647-52. Epub 2010 Mar 18.

Kirmizis A, Santos-Rosa H, Penkett CJ, Singer MA, Vermeulen M, Mann M, Bähler J, Green RD, Kouzarides T. Arginine methylation at histone H3R2 controls deposition of H3K4 trimethylation. Nature. 2007 Oct 18;449(7164):928-32. Epub 2007 Sep 26.

Dawson MA, Bannister AJ, Göttgens B, Foster SD, Bartke T, Green AR, Kouzarides T. JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. Nature. 2009 Oct 8;461(7265):819-22. Epub 2009 Sep 27.

1.10 PARTNER 10 - U Turku :

General summary In the first reporting periods of EPITRON, U. of Turku contribution was mainly focused on the analysis of HDAC functions in breast & prostate and other solid cancers, the impact of HDAC inhibitors on tumor cell proliferation, survival, and response of tumor subgroups like ETS-fusion gene positive prostate cancers that show high levels of HDAC1 expression, combined with other epigenetic players such as EZH2. Together with other partners in WP4, 7 and 10, we also analyzed gene expression changes in response to epigenetic drugs such as the ones developed by U. of Vigo in collaboration with Naples (Altucci/de Lera). Furthermore, within WP10 and WP7, we also had a strong contribution on the bioinformatic network analysis of TRAIL and related genes, focusing on the question of resistance versus sensitivity against TRAIL in particular. Also here, gene expression studies based on Illumina bead arrays were performed in collaboration with partner H. Gronemeyer. These arrays were normalized, analyzed and the resulting standardized data sets mined for gene co-expression networks. These results were compared to gene networks observed in solid cancers (using the IST database, now publicly available as GeneSapiens). At later reporting periods, we have shifted the focus from HDACs towards Histone Demethylases, partly in response to such activities in other partner labs. This was also partly based on our own screening resuslts that have pointed towards the HDM's as a key protein family with clearly tumor-relevant activities. Gene co-expression and network analyses have also been performed for histone demethylases and related genes. Identified as particularly interesting in prostate cancer, we have focused on members of the JmjC-domain containiing or Jumonji proteins; which covers about 30 members. Many of these genes have been very poorly defined, while others have been strongly associated with PrCa progression and in particular with androgen-receptor functions (e.g. JARID1A). Our studies have pointed to other members of this family as also interesting, including poorly studied genes like FBXL11, HSPBAP1 and PHF8. At later stages of Epitron, we furthermore engaged in chemoinformatic studies that were also geared towards identification of inhibitors that may inhibit key members of the JmjC family in cancer. This component is described in detail in the next paragraph. The activities within WP11 (bioinformatics) were initially mainly channeled into the development of the IST/GeneSapiens database of microarray studies, which featured a unique and novel normalization strategy that allowed to compare gene expression studies conducted by hundreds of independent researchers (on the Affymetrix platform) with each other, and to analyze expression of any given target gene of interest across hundreds of array datasets and thousands of individual arrays (n = 14800 at the time of reporting). These features were also trained and optimized based on epigenetic questions, e.g. expression of the HDAC family across human tumor entities and (most importantly) subtypes of tumors that may be defined by particularly strong epigenetic activities. This latter aspect is a key component of individualized medicine approaches, with the aim to identify tumors and tumor subtypes that are defined by strong activation of certain genes or pathways, including epigenetic players and their downstream signaling cascades. During later reporting periods for WP11, we have been engaging in chemoinformatics "virtual" screens with the aim to identify compounds that may inhibit the enzymatic activity of Histone Demethylases, using the JMJD2A protscaffold for these computational studies. These studies were also coordinated with the U. of Vigo (de Lera) lab. The resulting virtual hits were ordered from various suppliers, and utilized in functional cell-based screens to test for anti-proliferative

and/or pro-apoptotic activities. In parallel, these compounds were also analyzed in cell-free biochemical assays developed at Congenia, with the aim to identify specific inhibition of the enzymatic HDM function. Only some of the new compounds were active and also specific. However, initially none showed very strong anti-tumor effects in our validation experiments, using a panel of prostate cancer cell lines. Nevertheless, several interesting and promising scaffold structures were identified and subsequent chemoinformatic analog searches have resulted in more compounds that also have been screened for activity in both assays mentioned above. These compounds and scaffolds could be utilized to design improved compounds with stronger, specific activity. The histone demethylation inhibitor studies have not been completed yet, as some experiments concerning the downstream biological effects of cells treated with such inhibitors has not been finalized. Nevertheless, a manuscript has been drafted and circulated to the co-authors (de Lera, Mercurio), and will be pursued with new data added later this year. Furthermore, throuoghout the entire duration of Epitron, we have engaged strongly in "Demonstration Activity 3", which was related to high-throughput siRNA screens of epigenetic gene functions in malignant cancer cells. After initial approaches related to plate-based screening methods, we later shifted to the "cell spot microarray" or CSMA technology for primary screens, which allows 1000s of parallel, miniaturized cell-based assays utilizing a 100-fold smaller amount of siRNA. For this purpose, we designed an "epigenetic chromatinome players" library that contains siRNAs targeting 664 genes with approved or assumed epigenetic functions. Basis for gene selection (2006) was the then available information on protein domains with clear or assumed epigenetic functions (such as JumonjiC domain, SET and bromo domains, etc...), and also included genes identified by literature text mining (ClusterMed) with certified epigenetic functions. Also included were transcriptional co-activators and co-repressors and functional members of the key co-repressor complexes active in human cells. Using this siRNA library (n= 1350 siRNAs including controls), we eventually screened a large number of prostate cancer cell lines. The epigenetic library was also included in numerous screens based on other (breast, brain cancer) tumor cell lines, and also in cell-based screens using 384 well plates. Using novel image-based mining tools (developed as part of the bioinformatics activities in WP11), we were able to set up multiplex assays with up to 3 different antibodies as routine readout (e.g. AR androgen receptor, Ki67 cell proliferation, cleaved PARP for apoptosis). In the epigenetic cell based screens, we have additionally utilized a large panel of antibodies specific for methylated histone variants (H3K4me3, H3K4me2, H3K9me2, H4K16ac, etc..). These were complemented by DNA stain and actin staining (phalloidin) in routine screens. The image analysis tools developed for this task also made virtual FACS analyses possible, addressing simultaneously the number of cells in various phases of the cell cycle (DNA content) versus apoptosis or proliferation signals. As follow up studies to validate the findings from cell arrays, we have used ordinary 384-well plate based screeing, utilizing standard proliferation assays (CTG; Apo-1); and ordered independent siRNA sets for each one of the hits/genes under investigation (4 siRNAs per gene). On a third level of validation, successful siRNA silencing was monitored by Western blotting and qRT-PCR, prior to selction of a hit candidate for in depth functional profiling. Only very few genes (PHF8, HSPBAP1, FBXL11) were selected for further experimentation, which is in part (PHF8) still ongoing at the time of this reporting. U. Turku contribution during the last (5th) reporting period was mainly focused on the further development of the cell spot microarray technology and the associated bioinformatic mining tools, as well as the follow up of Histone demethylase

inhibitors, identified by chemoinformatic "virtual screens". Generally, the completion of ongoing studies has been our primary focus towards the end of the EPITRON funding period. We have restricted our follow-up studies on a very small number of targets. The biological function of the PHF8 gene, which was identified in a series of subsequent primary and secondary (cell based) screens, was further evaluated in a large panel of prostate cancers by immune histochemistry (collaboration with Tuomas Mirtti, Uiversity of Helsinki), using a large scale tissue microarray (TMA; with > 500 clinical samples) for this purpose. This clinical study has confirmed the upregulation of PHF8 protein in a subset of the hormone-refractory tumor. Currently, we explore the correlation with clinical parameters such as patient outcome, tumor grading, and lymph node/distant metastasis status. On the biological side, we have explored the impact of PHF8 over-expression and silencing on cell proliferation (plate-based screens and FACS), epithelial maturation and invasion of tumor cells (3D organotypic models), and gene co-expression networks (AR co-expression, ChIP studies on the AR promoter, immune cytochemistry). A manuscript has been submitted to "Oncogene" and is currently in revision. In parallel, we have also described the CSMA technology in a recent technical publication, which also covers the most recently developed data mining and processing tools involved. Last not least, a review article in "Epigenomics" described in detail the CSMA technology, with a special focus on epigenetic screens and their impact on understanding cancer biology. Furthermore, we have generally worked on improving the bioinformatics portfolio, adding a number of elements for bioinformatic analysis of gene expression data, specifically for mining large scale gene expression data sets that have become available only recently. The html portal "REX" which was devised to conduct such searches "online" without prior knowledge of programming, will be subject to a technical publication later this year as well. REX uses R-based scripts (bioconductor) that are embedded in an html environment and thus can be addressed via an internal server.

List of selected publications:

Härmä V, Virtanen J, Mäkelä R, Happonen A, Mpindi JP, Knuuttila M, Kohonen P, Lötjönen J, Kallioniemi O, Nees M. A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses. PLoS One. 2010 May 3;5(5):e10431.

Mari Björkman Päivi Östling, John-Patrick Mpindi, Juha Rantala, Tiina Vesterinen, Juha Turunen, Antti Rannikko, Ville Härm, Elisa Kaivanto, Pekka Kohonen, Matthias Nees and Olli Kallioniemi. Systematic knockdown of epigenetic enzymes identifies a novel histone demethylase PHF8 overexpressed in prostate cancer with an impact on cell proliferation, migration and invasion. *Submitted*.

Rantala JK, Mäkelä R, Aaltola AR, Laasola P, Mpindi JP, Nees M, Saviranta P, Kallioniemi O. A cell spot microarray method for production of high density siRNA transfection microarrays. BMC Genomics. 2011 Mar 28;12:162.

1.11 PARTNER 12 -Congenia:

During the entire duration of the project, Congenia was involved, in a collaborative effort with several different partners, in different workpackages and namely: WP2, WP5, WP6 and WP8. WP2: Congenia was involved in the activities related to the "Mechanistical analysis of chromatin dynamics and long-range/global modifications in the chromatin structure caused by PML-RAR". In the specific, Congenia supported partner 2 in the improvement and implementation of protocol for DNase I-hypersensitive sites (HSS), as subsequently, in the adaptation of methodology for the characterization of libraries of accessible sites (nuclease accessible sites, NAS). Ultimately Congenia contribute to the workpackage activities through an extensive and accurate statistical analysis of the results obtained in the characterization of HSS. Additional activities were done pointing up the attention on histone post-translational modification (PTMs). WP5: Congenia contributed to support partner 2a and 2b to conduct studies about "Tumor selectivity and mechanism of action of epi-drugs in Patient-matched breast and skin cancer models". On detail, Congenia contributed in the conditions optimization for handling and processing breast tumors primary cultures and to establish the conditions for the treatment of primary cultures with known epigenetic drugs. Congenia was also involved in the confirmation of data indicating that diverse human breast neoplasia are characterized by reduced levels of histone acetylation respect to normal mammary glands and that the levels of histone acetylation in a sample correlates with sensitivity to HDACi like VPA and TSA. Specifically, tumor samples with reduced histone acetylation basal level looked to be more sensitive to HDACi treatment compared to tumor samples with high histone acetylation basal level. Additionally, Congenia contributed to the conditions optimization for handling and processing melanoma tumors primary cultures. On details, it was established the conditions to run an automated proliferation assay and once established the best experimental conditions, the anti-proliferative activity of different histone deacetylase inhibitors as SAHA and LBH-589, in comparison with different chemotherapeutic largely used in clinical setting, (Temozolomide, Doxorubicin, 5-FU, Dacarbazine, CPT-11) was evaluated. The better antiproliferative activities observed with SAHA and LBH589, in comparison with different chemotherapeutic drugs, induced to characterize few selected melanoma patient derived cells looking at expression levels of different histone deacetylases, basal level of histone and tubulin acetylation and to analyze the molecular mechanis of action of different HDACi. The analysis of the molecular mechanism of action of selected Histone deacetylase inhibitors as Apicidin, MS275, FK228, SAHA, LBH-589, SB939, as representative of different chemical classes and different selectivity profile inhibitors, let to let to note, at least in these cells, that the inhibition of HDAC6 do not have a relevant contribution to the antiproliferative activity of the analyzed HDAC inhibitors. WP6: Initially, Congenia was involved in the attempt to produce crystal structure for several HDACs and more specifically HDAC class 1 enzymes. The following HDACs were prioritized: HDAC 1, 2 and 3, which are the main targets of HDACi action; HDAC8, for which the structure is already available, and therefore is more likely to give crystal structures in the presence of new inhibitors and HDAC11, which has been found to be deregulated in some types of cancer. With regards the crystallization efforts, unfortunately all the activities have not been successful at more than half of the Epitron grant period. For this reason, together with the Internal Management Committee and following the suggestions of the external scientific board of the Consortium (that has suggested phasing out activities for this part of the project); it was decided to

undertake new activities dedicated to establishment of an automated biochemical assay allowing the possible identification of histone demethylase inhibitors. For the scope, the effort of Congenia, in collaboration with European institute of Oncology and Second University of Naples, was therefore to produce active JMJD2a and JMJD3 recombinant proteins, to establish an automated biochemical assay allowing identify Histone demethylase inhibitors and subsequently to do two rounds of screening with compounds identified by University of Turku and Vigo . The chosen assay was a coupled–assay employing formaldehyde dehydrogenase (FHD), able to convert formaldehyde (released from histone lysine demethylation) to formic acid using NAD+ as the electron acceptor, whose reduction to NADH can be followed spectrophotometrically. With regard the establishment of the assay, at the first it were established the preliminary assay parameters as: reagent suitability and stability, linearity of enzymatic assay, signal to window, determination of initial velocity condition, determination of constant of affinity both for substrate and co-factor, maximal velocity (Vmax). Once identified the best conditions, it was therefore optimized all the process with a particular attention to the reproducibility and robustness of the assay, and ultimately to the automation of the established assay. Two subsequent round of screening testing around 200 compounds, were therefore conducted. The two rounds of screening let to identify two novel JMJD2a inhibitors. WP8: Congenia collaborated with partner 8 in order to achieve the task “Validation of intracellular receptor reporter mice (PPRE-Luc or ERE-Luc) for HDAC Screening”. Partner 8 generated reporter mouse models, using the knock-in strategy, in which it has been found that the bicistronic TKLuciferase cassette can be activated “in vivo” by histone deacetylase inhibitors. Congenia collaborated with partner 8 to verify the working hypothesis that PPRE-Luc or ERE-Luc reporter system present in reporter mice could constitute a biosensor of HDAC activity in vivo. Specifically Congenia, in collaboration with partner 8, was involved in the analysis of response of reporter system after topical application of a novel HDACi identified and supplied by Congenia. Simultaneously it was validate the level of penetrance into the derma of the histone deacetylase inhibitor dissolved in the topic formulations.

1.12 PARTNER 13- BSP:

SUMMARY ENTIRE CONTRIBUTION HDAC inhibitor project Two HDAC inhibitors, vorinostat and romidepsin, are on the market for the indication cutaneous T cell lymphoma. Numerous second generation HDAC inhibitors are currently being evaluated for improved efficacy and safety. One of them is the benzamide MS-275 (entinostat), a potent class I-selective HDAC inhibitor which is presently in clinical development for different tumor indications (hematological tumors such as leukemias and lymphomas, solid tumors such as non-small cell lung cancer, breast cancer and melanoma). The present study was performed to examine the effects of MS-275 in in vitro and in vivo models of human colorectal cancer. Treatment of five colon cancer cell lines with MS-275 at a sub IC50 concentration showed a graduated efficacy that did not correlate with changes in previously described pharmacodynamic markers such as p21 expression or H3 and H4 acetylation. The expression of HDAC2 in the cell lines correlated best with MS-275 efficacy in vitro. HDAC2 knock-down led to partial abrogation of the anti-proliferative effects of the compound. This isoform is therefore likely to be responsible for at least some of the anti-proliferative effects observed. On the other hand, additional cellular targets might be involved in the anti-tumor effects of MS-275, as the abrogation was not complete. HDAC inhibition by MS-275 did not lead to H3 and H4 hyperacetylation and p21 induction, the claimed hallmarks of HDAC inhibition, in all tested in vivo models of colon cancer. These changes can thus not be ascribed to every model of human cancer and every HDAC inhibitor. Our systematic analysis of different xenograft models as well as the in vitro analysis of five different colon cancer cell lines showed that there was a need for new, more sensitive and reliable biomarkers to predict the response and sensitivity of tumors to the treatment with MS-275. We therefore performed microarray analyses of colon cancer xenograft models to identify markers that distinguish between MS-275 responders and non-responders. This revealed a rather non-uniform response towards MS-275 treatment, which reflected the molecular heterogeneity of the xenograft models used. We did not see a correlation between the different types of xenograft models, either derived from established cell lines or colorectal cancer, and the response towards MS-275. We identified two responder cell lines (HT29 and HCT116) and one anti-responder (LoVo), two responder primary models (Co5854 and Co5676) and one non-responder (Co5776), as well as one metastasis model responder (Co6044) and one non-responder (Co5841). The gene expression profiles of these models were heterogeneous and the PCA revealed more differences among individual models than among treatment groups. We identified a number of potential biomarkers for MS-275 which were up-regulated dose-dependently upon MS-275 treatment in the five responder models and showed no induction in the three non-responder models. PRA1 is a ubiquitous protein which binds to prenylated Rab GTPases. Its overexpression impairs TCF/beta-catenin signaling, which plays a prominent role in colon cancer. MYADM is associated with the differentiation of hematopoietic and acute promyeolocytic leukemia cells. All-trans retinoic acid treatment induces expression of this differentiation marker in NB4 cells. The up-regulation of this gene upon MS-275 treatment may reflect the potential of the compound to induce differentiation in tumor cells and could serve as a differentiation and response biomarker for MS-275. Little is known about PALM2-AKAP2. The corresponding mRNA is a naturally occurring co-transcribed product of the adjacent PALM2 and AKAP2 genes but the function of its putative protein product has not yet been determined. A comparison of expression profiles of different HDAC

inhibitors revealed a very small overlap of regulated genes, pointing out to the importance of specific studies for each HDAC inhibitor when it comes to evaluating and validating stratification biomarkers. We studied the effects of MS-275 in eight different systems and found the overlap between regulated genes to be rather small, suggesting each model to carry individual patterns of epigenetic marks that contribute to their differential gene expression and response to treatment. Pathway analysis revealed that MS-275 strongly affected cell adhesion pathways. Among the 100 most significantly affected pathways, 20 were involved in the regulation of cell-cell adhesion, communication, motility, or tumor invasion. Several cell adhesion genes were up-regulated in MS-275 responder models but down-regulated in non-responder models. In the HT29 and HCT116 models many integrins and tetraspanins, such as beta1-integrin, CD9 or CD81, were up-regulated, whereas these genes were down-regulated in Co5841, Co5776 and LoVo. Besides that, E-cadherin, an important suppressor of epithelial tumor cell invasiveness and metastasis which is epigenetically silenced through promoter methylation in many carcinomas, was down-regulated in the non-responder models Co5841 and Co5776 upon MS-275 treatment. This implies that MS-275 did not induce the re-expression of E-cadherin, but rather promoted the hypermethylated status of the primary non-responder models. E-Cadherin as well as claudin 4 (CLDN4), which were down-regulated in Co5776, are decreased in diffuse type and poorly differentiated tumors. Dysfunction of these proteins may therefore play a role in disruption of cell-cell adhesion. In conclusion, MS-275 might increase the adhesive properties of tumor cells in responder models thus preventing the metastatic spread and the immune escape of the malignant cells, whereas in non-responder models most cell adhesion molecules were down-regulated. Taken together, the different in vitro and in vivo effects of MS-275 as well as the expression changes induced in responder and non-responder models revealed the pleiotropic effects of this compound on different cellular pathways affecting tumors and their microenvironment. PRMT project The protein arginine methyltransferase (PRMT) family includes several enzymes able to transfer a methyl group from adenosylmethionine to the guanidine nitrogen of arginine residues in target proteins. PRMTs are able to methylate N-tails of histones modulating thereby gene expression. PRMT1 and CARM 1 (PRMT4) have been described as cofactors for nuclear receptors, including the androgen receptor (AR). We therefore first wanted to analyze the role of the PRMT family in prostate cancer cell lines. The expression levels did not significantly differ between the cell lines analyzed. PRMT 1, 2, 5 and 9 showed high expression levels whereas PRMT 3, 6 and 7 showed much lower expression. PRMT 8 was not expressed in the prostate cell lines since this enzyme has been reported to be brain specific. Then we set up to investigate the role of these proteins in the proliferation of the different prostate cancer cell lines. A strong and specific reduction of mRNA levels for all PRMTs was achieved by siRNA-mediated knock-down. For PRMT1, 4, 5 and 6, for which antibodies were available, we could also shown a strong reduction of protein levels. In the androgen-dependent LNCaP cells we observed some anti-proliferative effects after down-regulation of PRMT9 and also very slightly effects after down-regulation of PRMT 1 and 7. In the VCaP cells, which are also androgen-dependent, no significant differences in proliferation after knock-down could be measured, but in this case the down-regulation of the PRMTs was only of about 40 – 60% which may not have been sufficient. In the PC-3 cell line, which do not express the AR, strong anti-proliferative effects were observed after down-regulation of PRMT5 and 9, and less pronounced effects after down-regulation of PRMT6 and 7. In order to investigate if these anti-proliferative effects were

specific for cancer cell lines, we also tested an immortalized, non-tumorigenic prostate cell line, RWPE-1. Only minor anti-proliferative effects were seen after PRMT5 down-regulation and no effects after down-regulation of the other PRMTs. We also tested the expression of several AR target genes in LNCaP cells after PRMT knock-down. Some reduction in the expression of PSA, TMPRSS2 and FKBP51 was observed after PRMT5 knock-down, however this was observed in the presence and in the absence of R1881. For PRMT5, only few target genes have been described. We tested the effect of PRMT5 reduction on the expression of ST7, NM23 and E-Cadherin in PC-3 cells and observed an increase in E-Cadherin expression after PRMT5 down-regulation whereas ST7 or NM23 expression did not change. Since PRMT5 is supposed to be the main enzyme catalyzing the symmetric di-methylation of arginines we tested the global levels of this modification with a specific antibody in PC-3 cells after PRMT5 knock-down. A strong reduction of the signal for symmetric di-methyl arginine was observed in the PC-3 cell extracts. Since PRMT5 knock-down displayed the most interesting effects, the role of gene was also analyzed in cell lines derived from other tumor types. Other members of the PRMT family are involved in estrogen receptor regulation and therefore we performed knock-down studies in an ER-positive cell line (MCF-7) and in an ER-negative cell line (MDA-MB-231). The proliferation assays performed with these breast cancer cell lines showed PRMT5 knock-down to lead to reduced proliferation and induction of apoptosis for MCF-7 cells (ER positive) but not for MDA-MB-231 cells (ER negative). In order to investigate a possible correlation of the anti-proliferative effects with ER expression, two additonal ER-positive cell lines were used, T47D or BT474 cells. Here, only a minor reduction of cell proliferation and no apoptosis induction was observed. No correlation between the anti-proliferative effects of PRMT5 knock-down and the expression of the steroid receptors could therefore be established. Other tumor cell lines were then selected for analysis, based on their high expression of PRMT5. Knock-down in the melanoma cell line A375 led to strong anti-proliferative effects. No comparable effect was observed in the lung cell line A549. Slight anti-proliferative effects were seen in the non-tumor cell line HaCaT. Altogether the knock-down experiments suggest PRMT5 to be involved in the proliferation of several cancer cell lines. First overexpression studies were not conclusive as high PRMT levels could not be obtained and will need more sophisticated approaches such as rescue experiments or co-transfection with additional, limiting cofactors (e.g. MEP50). The previously published inhibitors AMI-1 and AMI-3, and several putative new PRMT inhibitors synthesized in the laboratory of partner 5 were tested at a 100 µM concentration. Using PRMT1 in a methylation assay, inhibitory effects of AMI-1 and AMI-3 were seen, as previously published. The individual enantiomers of the described PRMT1 activator AMA-1 were tested as well. Surprisingly, AMA-1R and AMA-1S each activated PRMT1 methylase activity. Among the newly synthesized UVI compounds, UVI6001, UVI6003 and UVI6005 were the most potent inhibitors of PRMT1. In the PRMT5 enzymatic assay, AMI-1, AMA-1R and AMA-1S displayed activating effects. UVI6006, UVI6007 and UVI7514 were strong inhibitors. Concerning isoform selectivity, UVI6001 inhibited mainly PRMT1 and UVI6007 and UVI7514 mainly PRMT5. Proliferation assays were performed with all compounds on two prostate cancer cell lines. In VCaP cells, the best effects were observed for AMA-1R, AMA-1S, UVI7514, UVI7515 and UVI7516 with IC50 values in the range of 2.7 to 4.9 µM. In LAPC-4 cells, AMA-1R, AMA-1S and UVI7515 had inhibitory effects in the range of 0.6 to 1 µM. No apoptosis induction was observed for any compound in either cell line. Altogether, the results show that compounds that selectively inhibit PRMT1 or PRMT5 can

be identified. The correlation between PRMT1 or PRMT5 inhibition and anti-proliferative effects was not strong, however UVI7514 had the most promising profile since it strongly inhibited PRMT5 enzymatic activity and the proliferation of VCaP and LNCaP cells.

List of selected publications:

Bracker TU, Sommer A, Fichtner I, Faus H, Haendler B, Hess-Stumpp H. Efficacy of MS-275, a selective inhibitor of class I histone deacetylases, in human colon cancer models. Int J Oncol. 2009 Oct;35(4):909-20.

1.13 PARTNER 16 - Diagenode:

1. Objective Following Abcam's withdrawal from the EPITRON project and the consecutive Diagenode's take over, the objective for Diagenode in the EPITRON consortium was the generation of 150 antibodies against modified and unmodified epigenetic targets. These antibodies were to be used by the different partners in the different workpackages. 2. Procedure The procedure consisted of the following steps: - the antibody was requested by the EPITRON partner and approved by the project coordinator. - peptides were designed by the requesting partner and/or by Diagenode. - peptides were synthesized. - rabbit immunizations were started using standard procedures. - the different rabbit bleeds were sent to the partners for preliminary characterisation. - if requested by the partner the bleeds that passed the QC were purified using peptide affinity purification. - the purified antibodies were again sent to the requesting partner for characterisation. - efforts were undertaken to gather the characterisation results from the different partners. 3. Peptide design an immunization. Following the partner's request for the generation of an antibody and approval by the EPITRON coordinator, one or two immunogenic peptides per target were designed. The total number of peptides designed for the EPITRON project was 207. Approximately 55 of these peptides were designed by Diagenode, whereas the remaining peptides were suggested by the requesting partner itself and reviewed by Diagenode's Antibody Production & QC team. After synthesis and coupling of the corresponding peptides to the KLH carrier, the immunization was initiated using the standard procedure previously developed by Diagenode, working with a team of skilled veterinarians in a modern and ethical SPF (Specific Pathogen Free) animal facility. Two rabbits (New-Zealand white) were immunized for each target and each rabbit was injected 6 times with 200 µg of KLH-coupled peptide. Six bleeds were taken from both rabbits: a small bleed of 2 ml at Day 0, which served as a negative control; a second small bleed at Day 38, which was used to monitor the immune response; 3 large bleeds of 20 ml at Day 66, Day 87 and Month 4; and a final bleed of 50 ml after 4.5 months. Only the 4 large bleeds were sent to the requesting partner for preliminary characterisation. Using this standard procedure, a total of 150 antibodies against different modified and unmodified epigenetic targets have been produced. These antibodies were produced for the following workpackages: - WP1: 34 antibodies - WP2: 5 antibodies - WP3: 17 antibodies - WP4: 84 antibodies - WP11: 10 antibodies To generate these 150 antibodies, 300 rabbits were immunized and 1800 bleeds were taken of which 1200 bleeds were sent to the different partners for characterisation. 4. Purifications Affinity purification of the bleeds was offered as an option for those bleeds which passed the preliminary characterisation. Once the bleeds passed this QC, the partners could request from Diagenode the purification of those antibodies that performed at a satisfactory level in the desired application(s). These purifications were performed using affinity chromatography on an ÄktaPrime system from GE HealthCare against the peptide(s) used for the immunization. After purification an ELISA was performed as a Quality Control. Affinity purification was requested by the partners and performed for 21 of the EPITRON antibodies. The majority of these purifications (18) were performed for antibodies of WP4, whereas 3 antibodies from WP3 have been purified. After purification, the corresponding purified antibodies were again sent to the requesting partner for further characterisation. The partners involved in WP 1, 2 and 11 did not request any antibody purification. 5. Characterisation Efforts were undertaken to gather from the different partners their characterisation results for the crude

antisera and for the purified antibodies. Characterisation results for 69 antibodies were collected from the requesting partners, whereas two additional antibodies were characterized by Diagenode. Out of these 71 antibodies, 5 were tested in dot blot, of which 4 passed the QC; 55 antibodies were tested in western blot, of which 28 gave good results, and 17 were tested in immunofluorescence, all of which performed fine. Finally, out of the 29 antibodies that were tested in ChIP, only six turned out to be ChIP-grade, and one of these appeared to be also ChIP-seq grade. In total, 30 antibodies were performing well in at least one of the above mentioned applications. It has to be noticed that the characterisation of the remaining antibodies is still in progress at requesting partner's premises. All the antibodies generated for the EPITRON project are available for the different partners. Two of the characterized antibodies are currently available from Diagenode.

2. EPITRON Management team

This section summarizes the specific activities of the consortium's management team, which have been described in detail in the corresponding periodic reports.

2.1 Summary of main activities

The EPITRON Management team was set up to assist the coordinator in taking care of the scientific, administrative and financial follow-up of the project.

In agreement with the FP6 financial guidelines, the coordination was in charge of receiving and distributing the EC pre-financings among all the contractors. In addition, assistance was provided to the partners in dealing with any administrative or financial aspects related to the EPITRON project, and functioned as an intermediary between the EC and the contractors.
The development of the EPITRON workplan was accompanied by several changes in the composition of the partnership, redefinition of objectives and experimental strategies which resulted in the redistribution of tasks, effort and financial resources. The management team has coordinated the implementation of all these modifications and took care, whenever necessary, of communicating them to the Commission and making the corresponding amendments to the contract. These modifications include:

-The SME Abcam, who was in charge of producing antibodies for the EPITRON partners, stepped out towards the end of period 2 as a result of a restructuration of the company which rendered it unable to fulfil its obligations for the consortium within a suitable time frame. This task was taken over by DIAGENODE.

-The SME Epigenomics stepped out of the consortium as a result of re-defining some tasks, which resulted in giving up the use of bisulphite sequencing to adopt other technologies such as ChIP-chip and ChIP-seq.

-Discontinue the crystallization tasks undertaken by Congenia, after several unsuccessful attempts, and redirect the effort and financial resources of this SME to complement the drug discovery and validation pipeline.

-Invest more effort and financial resources on the drug discovery activities, which constituted the core of the consortium and lead to the synthesis of several compounds with anti-tumor action, one of them has been patented and its licensing to a pharmaceutical company is currently being arranged.

The meetings of the Executive and Internal Management Committees were organized by the EPITRON coordination. The aim of these meetings was to discuss all the issues relevant to the implementation and progress of the workplan as well as the administrative and financial aspects of the project. The corresponding minutes have been distributed among the contractors as well as to the scientific officer at the EC, they are also available at the EPITRON website www.epitron.eu. This website was established at the beginning of the

project and was updated regularly. It contains information about the consortium's objectives and composition, it also provides a space to advertise scientific events and open positions and contains as well an Intranet section with restricted access, in which all the documents relevant to the project can be found: contract and amendments, consortium agreement, meeting minutes, periodic reports and other information. In addition, the website provides a link to the EPITRON on-line reporting tool at http://report.epitron.eu . This tool has been conceived and developed entirely by the EPITRON Coordination with the help of an informatician who was especially hired for this purpose. Each partner had a user name and password to access the reporting tool in which three different types of reports were to be filled in: a scientific report, a financial report and a dissemination report.

After signing in, the main page of the program presents the basic instructions on how to fill in the reports. In addition, a tutorial video is available by clicking on the "Help" menu. This video guides the user through the different steps required to complete all the reports. There is also a small explanation available for the different sections to be completed, upon clicking on the following icon. Once the reports are completed, they are submitted for validation by the coordination. The coordination can accept the reports or re-send them to the partners for modification. Throughout the different reporting periods, this tool has been revised and improved taking into account the feedback from the partners and the experience from the management team. In addition, adjustments have been made to also be able to collect the necessary information for the preparation of the final report. All in all, we can conclude that this tool has facilitated the reporting process both for the partners and the coordination. It would be worthwhile to explore the possibility of making this software available to other users.

The EPITRON training program was implemented by the coordination and consisted in providing financial support to young investigators of the EPITRON labs to travel to scientific conferences or to travel to another EPITRON lab to perform experiments in collaboration and/or learn new techniques. The EPITRON Brainstorming meetings were also a part of the training program. These events were meant to bring together the EPITRON partners with leading experts in different fields related to the scopes of the consortium, discuss about the latest advances in those fields, exchange expertise, ideas and establish new collaborations. Travel grants were awarded to young researchers from EPITRON and external labs to attend these events and present their results in a special session dedicated to them. This was an opportunity for them to meet experts and discuss in a relaxed and private atmosphere. In some cases these interactions also resulted in new job opportunities for some of the young scientists. Further details on the grants provided and meetings organized in the context of the EPITRON training activities are included below in the attachments section.
The EPITRON coordination has taken into account the consortium's gender action plan when implementing the training program. Whenever possible, priority was given to women

researchers when allocating the travel grants. We tried as well to have a balance representation of genders in our panels of speakers at the EPITRON Brainstorming meetings.

2.2 EPITRON Brainstorming Meetings: Scope and invited experts

The EPITRON Brainstorming meetings were part of the consortium's training program. Their purpose was to bring together the consortium partners with leading experts in several fields related to cancer for discussing the latest advances in research, exchange ideas and establishment of possible collaborations. The meetings were closed with a small number of participants to favor interactions.

2.2.1 "Epigenetics and Cancer" Chania, Crete. September 10-12, 2007.

The purpose of this event was to bring together scientists working on the fields of epigenetics and cancer in order to discuss about the contribution of epigenetic processes to tumorigenesis and the potential of considering them as targets for new therapies.

Invited experts

Fyodor Urnov (Advanced Genomics Sangamo BioSciences, Inc. USA)
Deborah Bourc'his (INSERM U741, University Paris. France)
Rolf Ohlsson (Dept of Development & Genetics, Evolution Biology Centre. Sweden)
Wilhelm Krek (Institute of Cell Biology-ETH. Switzerland)
Bohdan Wasylyk (IGBMC, France)
Chi Wai Eric So (Haemato-Oncology Section, The Institute of Cancer Research. UK)
Luciano Di Croce (ICREA and Center for Genomic Regulation. Spain)
Ramin Shiekhattar (ICREA and Center for Genomic Regulation. Spain)
Kristian Helin (Biotech Research and Innovation Centre. Denmark)
Giulio Draetta (Merck Research Laboratories Boston, USA)
Michael Rosenfeld (Howard Hughes Medical Institute. University of California. USA)
William Hahn (Department of Medical Oncology, Dana-Farber Cancer Institute. USA)

2.2.2 "Apoptosis, senescence and stem cells in cancer". April 27-29, Budva, Montenegro.

The purpose of this event was to bring together scientists working in the fields of apoptosis, senescence and stem cells in relation to cancer. The aim was to exchange ideas and discuss concepts that are important for the development of neoplastic diseases and can lead to novel avenues towards paradigms for therapy. The fields of (cancer) stem cells, senescence and apoptosis are intimately linked and bringing together experts from each one is important to understand how alterations in these physiological processes contribute to cancer development.

Invited experts

Maarten van Lohuizen (The Netherlands Cancer Institute, Netherlands)
Bruno Péault (Stem Cell Research Center, Children's Hospital of Pittsburgh, USA)
Freddy Radtke (Swiss Institute for Experimental Cancer Research, Switzerland)
Jonathan Licht (Robert H. Lurie Comprehensive Cancer Center of Northwestern University, USA)
Claus Nerlov (EMBL Monterotondo, Italy)
Judith Campisi (Ernest Orlando Lawrence Berkeley National Laboratory, USA)
Peter Adams (The Beatson Institute, Glasgow Centre for Cancer Research, UK)
Daniel Peeper (The Netherlands Cancer Institute, Netherlands)

Patrick Mehlen (Centre Léon Bérard, France)
Boris Zhivotovsky (Karolinska Institute, Sweden)
Clemens Schmitt (Max-Delbrück-Center for Molecular Medicine and Charité, Germany)
Andrew Thorburn (University of Colorado Comprehensive Cancer Center, USA)

2.2.3 "Towards Epigenetic Treatment of Neoplastic Disease" Athens, Greece. March 24-25, 2011

The aim of this meeting is to get together with other experts and discuss the latest discoveries in epigenetics, focusing on their possible exploitation for the development of new therapeutic strategies for cancer. The success of our previous Brainstorming Meetings has encouraged us to maintain the same format, bringing together a limited number of leading experts and the EPITRON partners for the presentation of their latest results, exchange of expertise, ideas and intense discussions on the current status and perspectives of the epigenetics field in relation to cancer diagnosis, treatment and prognosis.

Invited experts
Andreas Ladurner (EMBL.Germany)
Victoria Richon (Epizyme, Inc.USA)
Ari Melnick (Weill Cornell Medical College.USA)
Ingrid Grummt (DKFZ.Germany)
Alexander Pintzas (Institute of Biological Research and Biotechnology. Greece)
Christopher Glass (University of California. USA)
Udo Oppermann (Botnar Research Centre.UK)
Wang Jun (BGI.China)