

**Executive summary:**

Cancer is one of the major causes of mortality. Epithelial cells become malignant after accumulating genetic mutations followed by morphological changes in the epithelium. Alterations in DNA include stable genetic changes in oncogenes, tumor suppressor genes and reversible epigenetic changes. Different forms of epigenetic mechanisms have been shown to modify the expression of key genes during tumour progression. Promoter DNA hypermethylation of tumour suppressor genes or DNA repair genes, and covalent histone modifications appear in early stages of neoplasia. Methods to identify early markers in different types of cancer are being developed, although very few are specific and sensitive enough to be applied in the clinic.

The aim of the present consortium EPIDIACAN was to develop sensitive and specific methodologies to identify early epigenetic markers for major types of cancer, like prostate and colorectal cancer. This project is based on recent findings that selected covalent histone modifications and their modifying enzymes can be early markers of tumourigenesis.

For this purpose, the following are applied:

- a) selected covalent histone modification like acetylation, methylation, phosphorylation, ubiquitination among others
- b) their modifying enzymes, like histone (de)acetylases, (de)methyltransferases
- c) appropriate diagnostic methods and tests for detection of selected markers in clinical samples.

During the first period the first focus of EPIDIACAN was on the development of experimental tools and models to detect and study histone modifiers as well as to develop sensitive methodologies for evaluation of their use in preclinical testing. The second EPIDIACAN focus for the first period was on launching non-invasive diagnostic methods for epigenetic modifiers based on technologies developed in the participating organizations to be tested in clinical samples. Appropriately selected clinical samples are utilized according to EU and national ethical procedures. During the second period the first focus of EPIDIACAN was to analyse the biological function of the selected histone modifiers and their target genes in colorectal and prostate cancer. The second EPIDIACAN focus for the second period was on the characterisation, validation of the above epigenetic markers for clinical applications using human prostate and colorectal cancer biopsies. Appropriately selected clinical samples are utilized according to EU and national ethical procedures.

The participants' relevant research activities during the second period resulted in 58 publications in high impact scientific journals. The members of the network also presented the major results in scientific conferences. Interaction between the network participants was intense, which was realized via short-term visits, the annual meetings of the network, bilateral meetings, via ftp site of the network and via electronic communications. The project objectives have been advertised in the press (newspapers).

The project's web site is at:

<http://www.eie.gr/nhrf/institutes/ibrb/eu-projects/epidiacan/index-en.html>

## **Project Context and Objectives:**

### **Concept of the project**

In the last few years our understanding of the complex mechanism of tumour progression has been dramatically changed by the identification of new mutated genes, which contribute in the multistep process and clonal expansion of tumours. In particular, genetic alterations of oncogenes and oncosuppressor genes have been closely associated with carcinogenesis. As a consequence, oncogenes/oncosuppressor genes contribute to the development of numerous aspects of the malignant phenotype by promoting cell cycle progression, resistance to apoptotic stimuli, neo-vascularisation and tumour metastasis. On the other hand, in recent years due to the newly developed powerful technologies of sequencing and analysis of gene regulation at the chromatin level, novel epigenetic mechanisms and factors related to cancer tumour progression have been identified. It is of interest that many of these epigenetic events appear early on the onset of cancer development and they can potentially be excellent early tumour markers. Indeed, a small number of epigenetic modifications have been tested already as early tumour markers, with very promising results.

Different forms of epigenetic mechanisms have been shown to modify the expression of key genes during tumour progression. DNA hypermethylation of promoter regions plays a role in silencing tumour suppressor genes or mismatch repair genes during tumourigenesis. Global DNA hypomethylation is observed in cancer and DNA hypomethylation at individual genes activate oncogenes in colorectal cancer. Demethylation in satellite sequences has been described as responsible to chromosomal instability in colorectal cancer. Further, loss of gene imprinting, a process mediated by DNA methylation and covalent histone modifications, is another epigenetic change that appears in the earliest stages of colorectal neoplasia causing abnormal gene expression. Finally, covalent modifications of histones like acetylation, methylation or phosphorylation, among others, distributed along promoters, coding regions and intergenic regions affecting chromosome condensation. Moreover, the cloning and functional characterization of histone modifying enzymes provides valuable information on the role of these epigenetic modifier factors. Although a lot of evidence has been accumulated for the role of epigenetic events and factors early in tumour progression, very few diagnostic applications in the clinic have so far emerged based on epigenetic markers.

The major challenge of this project was the development of new epigenetic markers for major types of cancer, like prostate and colorectal cancer that will allow early detection of tumour cells in patients, and will have important implications for successful therapeutic strategies in individual patients. A better mechanistic understanding of key epigenetically regulated genes involved in tumour progression in vivo will also help to develop tests for later clinical development and use. The key concept of this project was to develop diagnostic tools for the detection of epigenetic modifiers whose function has been implicated in cancer as potential tumour biomarkers.

The focus of the proposed collaborative effort was on:

1. The analysis of Usp22, SMYD3, LSD1, JMJD2c, PRK1, JMJD3 and EzH2 function in newly developed cell culture and animal models.
2. Identification of their downstream target genes to understand their role in tumourigenesis and to discover novel potential tumour biomarkers.

3. Development of efficient technologies for sensitive and specific epigenetic marker detection:

- a. characterisation, validation of the above epigenetic markers for clinical applications using human prostate and colorectal cancer biopsies.
- b. Characterisation, validation of the above epigenetic markers for clinical applications using circulating cancer cells obtained by non-invasive methods.

EPIDIACAN was carried out in close collaboration between investigators having complementary and multidisciplinary expertise in cell and molecular biology, epigenetics, biochemistry, tumour biology, clinical studies and tumour marker technologies. The studies of this Network have been directed to major aspects of cancer in model organisms, in mammalian systems and in clinical samples. This network composed of such teams provided unique and challenging opportunities for researchers interested in different aspects of epigenetics and cancer by using modern and developing new technologies to achieve their goal. It also provided the participant organisations, especially the small and medium-sized enterprise (SME), with epigenetic markers to be further exploited as industrial applications.

## **Project objectives**

1. Generation of experimental tools (antibodies) and models to study epigenetic modifiers and for use in preclinical testing of epigenetic biomarkers.
  - a. Development of specific antibodies for the detection of selected histone modifiers (HM)
  - b. Generation of cell culture and animal models (KO and transgenic) for studying histone modifiers function.
  - c. Identification of target genes regulated by HM by global gene expression profiling and by ChIP-seq-based global occupancy analysis in the cell culture and animal models.
  - e. Identification of stage specific and oncogene specific epigenetic alterations in model systems of prostate and colorectal cancer.
  
2. Development of novel diagnostic tools for the detection of epigenetic modifiers and modifications in clinical samples.
  - a. Development of diagnostic tools/methods for the detection of selected modifiers including expression in tumour biopsies.
  - b. Development of diagnostic tools/methods for the detection of selected downstream targets of HM in tumour biopsies.
  - d. Development of improved non-invasive technologies for the isolation of cancer cells from body fluids that allow early detection of cancer.
  - e. Evaluation of diagnostic tools/methods detecting modifiers in cancer cells isolated from body fluids.

**Project Results:**

**The major achievements of this period are as follows:**

1. Genome-wide occupancy analysis performed by ChIP-sequencing with global expression profiling data and application of comparative analysis, retrieved JMJD2C, LSD1 target genes such as EGFR, CDK1 or the PRK1 target KLK2 and KLK3.
2. In PC3 cells we detected 488 and 1355 genes differentially regulated by LSD1 and PRK1, respectively. In contrast the PRK1 controlled gene set is associated with migration and invasion.
3. SILAC screens were performed for proteins interacting with LSD1 and JMJD2C respectively, and several novel interactors among members of the CoREST and NURD complex have been characterized
4. Our analyses reveal that the protein kinases CDK5 and PKA are able to phosphorylate LSD1 at position T119. Phosphorylation of LSD1 at position T119 does not alter demethylation or complex assembly.
5. An important role of Smyd3 in liver and intestinal cancer was identified. The mechanistic basis of its function involves promotion of epithelial-mesenchymal transition. Smyd3 was not recruited into the regulatory regions of S-phase specific genes. Target genes most relevant to the carcinogenesis process were those regulating EMT. These include members of the matrix metalloprotease family.
6. EZH2 silencing de-represses EMT related genes and affects cell migration and anoikis in colon cancer cells. ITGA2 has been identified as a novel EZH2 target gene and was further validated. ChIP-sequencing analysis has further identified new EZH2 target genes.
7. EzH2 levels are associated with cancer stem cells (CSCs) in our cultures, and EzH2 was down regulated when the CSCs differentiated This has been confirmed by showing that knocking down EzH2 activity using siRNA increases CSC differentiation
8. The tightly controlled deubiquitination activity of the human SAGA complex differentially modifies distinct gene regulatory elements. The structural plasticity of two different SCA7 domains of the SAGA deubiquitination module defines their differential nucleosome-binding properties. Deregulation of H2B ubiquitination may contribute to cancer development.
9. A total of 23 TMAs representing 1.242 tumor tissue samples in duplicate including pairs of primary tumors and metastases was generated in the reported period, from the following tumor indications: prostate, colon, breast, lung, kidney, stomach, head and neck, ovary, cervix and skin.
10. Collection of 240 fresh-frozen samples of prostate cancer tissue, as well as matched bodily fluids (urine and peripheral blood) has been performed, which have been stored at -80°C. Simultaneously, bodily fluids from 200 healthy donors and patients harboring other urological pathologies have also been collected and stored for further analysis. Collection of fresh-frozen samples of colon cancer tissue and storage for further analysis was performed
11. We found that SMYD3, an H3K4 methyltransferase, was significantly over-expressed in tumor samples mainly in advanced stage prostate cancer
12. The data from our analysis show that LSD1 is predictive marker of prostate cancer superior to PSA.
13. High EZH2 mRNA levels in human colorectal cancer (CRC) specimens mark metastatic disease. Higher EZH2 expression was observed in more invasive CRCs and CRCs with regional lymph node metastases, Higher H3K27me3 expression was observed in more invasive CRCs, CRCs with regional lymph node metastases and in CRCs at advanced stages of disease, as well as in

CRCs with lymph and venous vessel invasion, which appears to be associated with the putative role of EZH2 in the progression of CRC.

14. Lower LSD1 expression was observed in more invasive CRCs and in CRCs at advanced stages of disease, suggesting that LSD1 may be associated with less aggressive CRCs. Higher H3K9me3 expression was observed in CRCs with regional lymph node metastases, which appears to be associated with the putative role of EZH2 in the progression of CRC.

15. SETDB1 expression was observed in left-sided CRCs, suggesting a putative role in the carcinogenesis of CRCs with chromosomal instability.

16. Concerning response to treatment, higher survival rates in patients with CRCs treated only with surgery were found for tumours displaying lower SETDB1 expression, suggesting that whenever the expression of SETDB1 occurs, the surgical treatment may not be enough to prolong the patient's survival. Importantly, LSD1 expression and the histone mark H3K9me3 may predict the response of CRC patients treated with Folfiri, and H3K9me3 and H3K27me3 expression may predict CRC patients' response to 5-FU/Leucovorine.

17. F-actin (filamentous actin) is a useful marker of colorectal 1 (CRC) cell line derived lumens. Lumen formation is a feature of tumours in vivo, and human primary low and high grade colorectal tumours show differences in ezrin/actin and CEA polarisation. Lumens represent enterocyte brush border differentiation, but also contain secretory lineages. Polarised F-Actin is a marker of colonic brush borders in vivo and labels Matrigel grown lumens in vitro.

18. A new approach to establishing primary cultures from fresh CRC patient tumour material was developed.

## **Potential impact and use**

This consortium has assessed epigenetic factors and mechanisms, like histone modifications as well as modifying enzymes involved in these processes. Cancer models have been selected for the most part of the studies and results have been validated in clinical specimens. EPIDIACAN consortium contributes in identification of novel oncogene and tumour stage related epigenetic events and factors, in order to maximize the subsequent clinical exploitation as epigenetic markers. Candidate epigenetic markers as well as marker combinations have been tested in a panel of preclinical models (cell lines and transgenic mice). A tight management plan was adopted in order that the most efficient combination per tumour cell to reach the final evaluation in clinical samples. There is the challenge that all this basic mechanistic analysis of genetic vs. epigenetic pathways will be utilized to improve clinical practice and finally public health.

The benefits on the society of a network focusing on novel early tumour markers are evident, since cancer is a major cause of death in Europe. This network contained groups of scientists from different disciplines like Molecular Biology, Epigenetics, Cell Biology, Tumour Biology, Biochemistry and Clinical Studies.

Bilateral interactions enhanced already successful co-operation by adding a value at the European level. Dissemination of the technologies developed at the individual national level will provide patients all over Europe to have access to new developments concerning cancer treatment, thus enhancing the benefit for the "European Citizen".

Part of the work will therefore have direct implications on colorectal, prostate and breast cancer in humans through novel epigenetic markers for cancer diagnosis and cancer therapeutics. Further characterization of our already existing cell and mouse models for cancer on different genetic backgrounds allowed us to use these mice in preclinical trials and speed up the evaluation of novel markers in the clinic. New knowledge about tumour formation has eventually been translated to an application stage, to the design of new diagnostic tests to detect cancer at an early stage and thus improve medicine in Europe and life quality.

The project extended the service business model and thus increases the competitiveness of the participating Targos Molecular Pathology GmbH. The company had the opportunity to further explore own biomarker projects and build up own intellectual property within this project and to profit from the transfer of know-how from the collaboration with outstanding experts from different fields of life sciences and technology. Targos further improved its knowledge in the field of epigenetics on a tumour and patient specific manner and was able to use the network resources in order to improve its competitiveness in the market.

We have also developed a efficient method to grow primary tumours from biopsies and endoscopies and this has the potential to significantly improve diagnosis and tumour classification. The health implications of this approach are potentially large as anti-cancer treatments can now be applied to growing samples derived from a much wide variety of patients. There is scope that our approach may allow some personalised treatments to be tested in vitro for efficacy, for example, antibody mediated immune killing using the patient's own blood.

Furthermore in vitro studies of tumour differentiation may also be used to refine the classical pathological definition of differentiation. The potential cost benefit for EU healthcare may be substantial as drug treatments can be refined and suitable drug regimes tailored to suit each individual patient.

The prostate is the most common site of malignant transformation in western men. About 30% of men older than 50 years in the western countries develop prostate adenocarcinoma and the lifetime risk of clinical disease is 10%, with the risk of mortality being around 3%. The great variation in clinical behavior of prostate cancer creates a major dilemma in the treatment-decision process, since not all men with microscopic carcinoma require aggressive radical therapy. The major difficulty is the present-day absence of reliable tools to predict which cancers will remain indolent and which are going to kill the patient if left untreated. This project identified epigenetic markers of biological aggressiveness in prostate cancer and this information may have a wide impact on health and socioeconomics, both by reducing over-treatment and by allowing concentration of resources to treat intensively those patients who actually need it.

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A detailed description of the work performed by each contractor during the reporting period is included. This description is addressed at the workpackage level. Figures are included in the corresponding periodic reports.

Participant 1. Alex Pintzas, NHRF.

**WP1.** Antibodies against EzH2 and JMJD3, as well as against H3K27Me3 were tested for optimal conditions in assays like immunohistochemistry, western blot, immunofluorescence and Chromatin immunoprecipitation (ChIP).

**WP2.** Generation of colon cells with silenced EzH2. Effect of silenced (inhibited) PIK3CAmut oncogene on EzH2 expression. To determine the contribution of EZH2 over expression to malignant phenotype of CRC cells we silenced EZH2 using small interfering RNA oligonucleotides and a vector to stably over express short hairpin RNA versus EZH2 mRNA (siEZH2 and shEZH2 respectively). As the expression of EZH2 was highest in cell lines with EMT behavior we decided to destroy the EZH2 endogenous protein in HCT116, SW620 and CacoH2 cells with a pool of siEZH2 oligonucleotides. To characterize the phenotype of siEZH2-silenced CRC cells we first evaluated the migratory ability using transwell system. The results showed that transient impairment of EZH2 reduced the migratory ability of CRC cells. In fact, the number of CacoH2- and HCT116-siEZH2 cells compared to parental cells were 69% and 83% respectively. We then concentrated only on HCT116 cell line for stable over expression of shEZH2 vector. The characterization of shEZH2 HCT116 stable clones revealed that EZH2 protein was reduced greater than 80% and reduction on global H3K27me3. Furthermore, when shEZH2-HCT116 cells (HCTshEZ-2) were grown in Matrigel, 3D culture, they lost the ability to migrate as demonstrated by wound healing and migration assay (C and D).

Silencing of EZH2 derepresses EMT related genes in Caco-H2 cells. We reported a large list of genes related to EMT phenotype that were deregulated in Caco-H2 compared to parental Caco2 cell line (Joyce et al. 2009) as well as global and local histone post-translational modification pattern in the same cell models proposing that EZH2 might be a possible regulator of EMT (Mazon-Pelaez I. et al., 2010). The outcome of previous works and results obtained with siEZH2 let us to speculate that EZH2 really might be related to the invasiveness and migratory ability of CRC cells. To verify this hypothesis and in order to search

for putative EZH2 target genes we decided to compare a group of 30 selected genes, chosen based on previous results, in siEZH2 versus parental EMT cell lines. We initially performed the study using CacoH2 cell line as we had microarray data for this cell system and afterward a subset of the 30 genes was analyzed also in HCT116 and SW620 cell lines. We initially performed the study using CacoH2 cell line as we had microarray data for this cell system and afterward a subset of the 30 genes was analyzed also in HCT116 and SW620 cell lines. As positive control, E-cadherin gene has been identified as an EzH2 target gene. The gene validation is currently in progress. Deliverables 3.1 and 3.2 (scheduled for month 24) have not yet been achieved.

We observed that forced expression of RAS and BRAF oncogenes in Caco2 cells caused hyper activation of RAS-PI3KA-AKT pathways with concomitant over expression of EZH2 (Mazon-Pelaez I. et al., 2010). Silencing of PIK3CA or inhibition of the pathway by small molecule, Wortmannin a PI3KA inhibitor, reduced pAKT and provoked EZH2 down regulation

**WP3. Generation of colon cells with silenced EzH2. Effect of silenced (inhibited) PIK3CAmut oncogene on EzH2 expression. ChIP-sequencing analysis of Global EZH2 target genes.**

To determine the contribution of EZH2 over expression to malignant phenotype of CRC cells we silenced EZH2 using small interfering RNA oligonucleotides and a vector to stably over express short hairpin RNA versus EZH2 mRNA (siEZH2 and shEZH2 respectively). As the expression of EZH2 was highest in cell lines with EMT behavior we decided to destroy the EZH2 endogenous protein in HCT116, SW620 and CacoH2 cells with a pool of siEZH2 oligonucleotides. To characterize the phenotype of siEZH2-silenced CRC cells we first evaluated the migratory ability using transwell system. The results showed that transient impairment of EZH2 reduced the migratory ability of CRC cells. In fact, the number of CacoH2- and HCT116-siEZH2 cells compared to parental cells were 69% and 83% respectively. We then concentrated only on HCT116 cell line for stable over expression of shEZH2 vector. The characterization of shEZH2 HCT116 stable clones revealed that EZH2 protein was reduced greater than 80% as well as reduction on global H3K27me3 was achieved (A and B). Furthermore, when shEZH2-HCT116 cells (HCTshEZ-2) were grown in Matrigel, 3D culture, they lost the ability to migrate as demonstrated by invasion assay as well as the ability to move as demonstrated by wound healing assay (C and D).

Identification and characterisation of oncogenic signalling pathways that modify EzH2 in colorectal cancer EZH2 is regulated by ERK and AKT pathways via AP-1 transcription factor in colon cancer cells with EMT phenotype. Initially we observed that forced expression of RAS and BRAF oncogenes in Caco2 cells caused hyper activation of RAS-PI3KA-AKT pathways with concomitant over expression of EZH2 (Mazon-Pelaez I. et al., 2010). Silencing of PIK3CA or inhibition of the pathway by small molecule, Wortmannin a PI3KA inhibitor, reduced pAKT and provoked EZH2 down regulation. To better dissect the pathways involved in EZH2 regulation we assessed EZH2 expression before and after blocking ERK and AKT pathways using siRNA and two specific inhibitors, in Caco-2, Caco-H2, HCT116 and SW620 cells. pERK1/2 inhibition by the MEK inhibitor U0126 in Caco-2 cells resulted in 10% reduced EZH2 protein and mRNA levels. In Caco-H2 cells EZH2 mRNA and protein expression (by 49%) were significantly reduced after U0126 treatment. In SW620 cells, a considerable inhibition of pERK1/2 was observed after U0126 treatment with a consequent 30% reduction of EZH2 protein and mRNA levels.

In HCT116 cells no effect at EZH2 protein and only marginal on EZH2 mRNA level were detected, even though pERK1/2 levels were reduced. This may be due to the fact that HCT116 cells bear PIK3CA H1047R mutation and EZH2 expression might be sustained by oncogenic pathways involving AKT activation. This hypothesis is supported by the following data: Wortmanin, a PI3K inhibitor, efficiently blocked pAKT in HCT116 cells, whereas its effect on Caco-2 and overall on Caco-H2 cells was transitory. In HCT116 cells reduction of pAKT provoked EZH2 protein and mRNA downregulation by 50% after 4 h, con-firming that PI3K pathway is the major regulator of EZH2 in this cell line. Furthermore, in SW620 cells EZH2 mRNA, but not protein, reduction was documented after 4 h of treatment with Wortmanin. No effects on EZH2 expression in Caco-H2 cells either a 2 or 4 h with 1 'M Wortmanin treatment were observed. We used siRNA to silence AKT and both ERK proteins (ERK1/2). The results showed that EZH2 expression was affected by suppression of AKT and ERK1/2 in HCT116 and SW620, but no effects on EZH2 were detected in Caco-H2. This is likely due to the huge amount of pAKT and pERK1/2 present in Caco-H2 cell line. Finally, to further demonstrate that ERK and AKT pathways are able to control EZH2 expression, ERK1/2 and AKT hyperphosphorylation was induced in Caco-2 cells by transforming growth factor beta-1 (TGFb-1), FBS and the mutant form of Harvey-RAS oncogene. TGFb-1 and FBS treatments induced pERK1/2 and EZH2 protein expression by 35% and 30% respectively, whereas transient transfection of Harvey-RAS V12 oncogene induced AKT hyperphosphorylation resulting in increased expression of EZH2 protein by nearly 50%.

The genomic promoter region of EZH2 was studied in order to be demonstrated that ERK and AKT pathways control EZH2 via the well known nuclear effector AP-1 transcription factor. For this purpose, one thousand nucleotides (nts) upstream and 200 nts downstream the transcription start site (TSS) of EZH2 promoter were screened for the presence of transcription factors binding site using TFSEARCH software. Two putative AP-1 binding sites with high score were identified within the region screened. The first was located at position -772 nts upstream to TSS with a score of 84.6/100 whereas the second was located at position +75 nts downstream to TSS with a score of 81.5/100. Expression levels of two AP-1 components, FRA-1 and C-JUN, were examined in comparison to EZH2 expression in all cell lines. FRA-1 and C-JUN were found overexpressed in SW620, RKO and HCT116, that also presented high levels of EZH2. To validate the role of AP-1 transcription factor on EZH2 regulation, we silenced FRA-1 and C-JUN proteins in cell lines with EMT properties. siRNA oligos showed different efficiency in the three cell lines used. However, in SW620 and Caco-H2 cell lines, reduction of EZH2 protein levels was observed after transient reduction of FRA-1 and C-JUN. In HCT116 cells, silencing of FRA-1 and C-JUN did not affect EZH2 protein level, similar to the effect of MEK inhibitor treatments in this cell line. Furthermore, binding of AP-1 complex to EZH2 promoter was analyzed in different cell lines by ChIP analysis comparing the levels of FRA1 and C-JUN and their presence on EZH2 promoter. RKO, SW620 and HCT116 were selected, since they express high FRA-1 and C-JUN levels, whereas Caco-2 was used as control due to low expression FRA-1 and C-JUN levels. Enrichment of the region containing the AP-1 site at +75 was detected in HCT116, RKO and SW620 but not in Caco-2 cell line, confirming that the occupancy of EZH2 promoter by these AP-1 components was related to FRA-1 and C-JUN protein levels .

**Silencing of EZH2 derepresses EMT related genes.**

We reported a large list of genes related to EMT phenotype that were deregulated in Caco-H2 compared to parental Caco2 cell line (Joyce et al., 2009) as well as global and local histone post-translational modification pattern in the same cell models proposing that EZH2 might be a possible regulator of EMT (Mazon-Pelaez I. et al., 2010). The outcome of previous works and results obtained with siEZH2 let us to speculate that EZH2 really might be related to the invasiveness and migratory ability of CRC cells. To verify this hypothesis and in order to search for putative EZH2 target genes we decided to compare a group of 30 selected genes, chosen based on previous results (Joyce et al., 2009), in Caco-H2 siEZH2 versus parental the cell line. A subset of the 30 genes was then analyzed in the other two EMT cell lines available: HCT116 and SW620. Table S1 shows the results of qPCR performed on Caco-H2 cells. ITGA2, SPRY1, CDH17, Coll type II A1 and CCND2 were significantly up-regulated whereas CCND1 was down-regulated after transient reduction of EZH2 in Caco-H2. Downregulation of CCND1 was detected in all cell lines, whereas CCND2 and CDH17 were up regulated in SW620 cells and ITGA2 and Coll Type II A2 were up regulated in HCT116 cells. The analysis indicated that Caco-H2 cell line after EZH2 silencing presented a molecular mark which is a sum of the other two EMT cell lines and that ITGA2 was a potential EZH2 target gene. We compared the ITGA2 mRNA absolute levels in HCT116, SW620 and Caco-H2 before and after EZH2 transient silencing.

The comparative qPCR analysis shows that HCT116 exhibited the highest level of ITGA2 mRNA after EZH2 silencing, followed by Caco-H2 and SW620. WB assay confirmed that ITGA2 was de-repressed in HCT116 cells after transient and stable EZH2 silencing. In Caco-H2 cells de-repression of ITGA2 was only observed at mRNA level but not at protein level, whereas in SW620 cells ITGA2 expression was not affected by siEZH2. Treatment by EZH2 inhibitor DZNep confirmed ITGA2 de-repression in HCT116 and SW620 cells. Confocal microscopy observation showed increase of ITGA2 expression in HCTshEZ-2 and in transiently siEZH2-HCT116 cells compared to control cells. Finally, to discover whether an epigenetic mechanism led by EZH2 might control ITGA2 expression in HCT116 cells, ChIP analysis was carried out. Two couples of primers positioned at -700 nts and +40 nts from the TSS covering a large portion of ITGA2 promoter were designed. The occupancy of E-cadherin promoter by EZH2 was evaluated in parallel, as a control EZH2 target gene. Presence of EZH2 and H3K27me 3 mark on ITGA2 promoter was confirmed in HCTshpSUP and SW620 cells since enrichment with both couples of primers was obtained. By contrast, EZH2 occupancy and H3K27me 3 mark were remarkably reduced on ITGA2 promoter in HCTshEZ-2 cells. The same occupancy pattern in HCTshEZ-2 cells was observed on E-cadherin promoter adding further evidence that these two genes are direct targets of EZH2. In accordance with previous transient experiments, we obtained further evidence supporting the role of EZH2 in metastasis related properties. In fact, HCTshEZ-2 cells have lost the ability to migrate and invade as demonstrated by wound healing and invasion assays. Expression analysis of E-cadherin, an epithelial marker, and N-Cadherin, a mesenchymal marker, confirmed that reduction of EZH2 expression reverts at least in part the mesenchymal phenotype, since E-cadherin protein expression remarkably increased (by 2-3-fold) in HCTshEZ-2 cells compared to control cells, whereas no reduction was detected for N-Cadherin.

Most notably, when HCTshEZ-2 cells were grown in matrigel, apoptosis was detected. Indeed, while parental HCT116 cells and HCT116 cells stably transfected with vector alone (named HCTshpSUP) were able to form large tumor-like foci, HCTshEZ-2 cells grew in a single-cell monolayer, most

likely cells that abandon the basal monolayer undergo anoikis, apoptosis associated to cell detachment from the matrix. Hoechst nuclear staining, cleaved (active) caspase3 antibody and confocal microscopy were used for detection of apoptotic cells.

#### **ChIP-sequencing analysis of Global EZH2 target genes**

In order to best characterize the phenotype of HCTshEZ2-2 cells and search for new EZH2 targets related to cancer, we performed ChIP experiment followed by massive parallel sequencing (ChIP-seq). The template for sequencing was prepared using the anti-EZH2 antibody on sonicated chromatin extracted from HCT116 parental and HCTshEZ2-2 cells. For each cell line we performed sequencing in 2 biological replicates (HCT116\_B and HCT116\_109; HCTshEZ2-2\_A and HCTshEZ2-2\_B). The overall quality of sequencing was good even though an optimization of ChIP protocol is needed in order to confirm interesting preliminary results and cover some genomic loci that are poorly resolved.

The preliminary local analyses of bind sites on promoter elements of the known EZH2 target gene E-cadherin (CDH1) confirmed that the methyltransferase binds near the TSS of E-cadherin. Upon EZH2-silencing (HCTshEZ2-2) the promoter region of E-cadherin does not show enrichment or presence of any peak. These results are in line with the de-repression of E-cadherin in HCTshEZ2-2 cells. The local analysis of ITGA2 promoter was not possible due to the low quality of ChIP-seq data in that specific locus.

Interestingly we identified a new putative EZH2 target gene by comparing the peaks patterns on promoter elements between the replicates of the two cell lines. Indeed, few nucleotides upstream the locus of a gene named GLRX3 we found the same pattern present on E-cadherin promoter. Peaks were evident on the tracks of parental cells but completely absent on the tracks of HCTshEZ2-2 cells. In addition based on data reported by Encyclopedia of DNA Elements (ENCODE) project, the binding site on HCT116 parental cells are particularly close to the H3K4me3 mark often found near promoters. Moreover, referring to ENCODE data on GLRX3 locus the H3K27ac mark was particularly high indicating that this gene is expressed by normal cells.

Another interestingly result was found roughly in the middle of the long arm of chromosome 2 (q32.1). In this region the same pattern of peaks covering 70-80 kb was found in all cell lines analyzed. That region according to ENCODE data (H3K4me3 and H3K27ac) contains expressed genes both for proteins and microRNA. Of note, the microRNA is named mir663b and according to the literature is silenced in colon cancer cell lines such as HCT116, RKO and DLD1 (Yan H et al, 2011). The importance of this finding is that this putative EZH2 target region could play a crucial role on cancer. Indeed, the fact that this region is kept silenced also in HCTshEZ2-2 cells could mean that it contains elements potentially lethal for the cells, such as genes able to control cell proliferation or apoptosis.

#### **WP5. EZH2 mRNA levels in human CRC specimens marks metastatic disease.**

EZH2 mRNA expression was evaluated in 51 human CRC specimens by qPCR. Over-expression (greater than 1.3-fold) was found in 37.2% (19/51) of tumor samples whereas in 29.4% (15/51) EZH2 mRNA was less than 0.7-fold (down regulated) with respect to a pool of normal colon tissues. In 33.3% (17/51) of tumor samples no significant changes were detected. Specimens were grouped based on EZH2 expression and presence of lymph node and/or

distant metastasis. Even though the percent-age of specimens positive for metastasis was roughly the same in all three selected groups (68.4%; 62%; 66.7% respectively), in samples with EZH2 over-expression the magnitude of up-regulation was significantly higher in patients presenting metastasis. Fold-change average of EZH2 in this group was 2.83, whereas in samples devoid of metastasis was 1.67, close to the limit of 1.3 fold used for up-regulation. Mann-Whitney test (p-value = 0.043), performed on the two sub-groups with EZH2 over-expression, indicates that higher EZH2 mRNA levels correlate with presence of lymph node and distant metastasis.

JMJD3 mRNA level in human CRC. JMJD3 mRNA expression was evaluated in 49 human CRC specimens by qPCR. Over-expression (greater than 1.3-fold) was found in 34.7% (17/49) of tumor samples whereas in 32.6% (16/49) JMJD3 mRNA was less than 0.7-fold (down regulated) with respect to a pool of normal colon tissues. No significant changes were detected in 32.6% (16/49) of samples. Interestingly, in the group with F.C. greater than 1.3 the vast majority of samples presented metastasis 88.23%. The percentage of patients presenting metastasis was lowering as the expression of JMJD3 decreases. Indeed, in the group of specimens with no JMJD3 deregulation (0.7 less than F.C. less than 1.3) the percentage of patients positive for metastasis was 68.75% whereas in the group where JMJD3 was down-regulated (F.C. less than 0.7) the percentage of patients positive for metastasis was 50%. The study revealed a possible association between JMJD3 over-expression and presence of metastasis.

## **Participant 2. Sir Walter Bodmer, WIMM.**

### **WP2 Development of cell culture and animal models to study epigenetic regulators and modifications**

We have analysed the expression data for the 7 genes of special interest to the Epidiacam project on our colorectal cell line panel, using just over 90 of the cell lines. For only one of the genes, PRK1 (official name PNK1), is there a clear cut suggestion of two different groups of lines with different levels of expression. In the figure we plot the cell lines in rank order of expression and look for evidence of two or more groups of lines with different levels of expression by testing for non-linearity of the slope of the rank order plot expressed in terms of the expected ranks on the assumption of a normal distribution (a rankit plot). Otherwise the slope of such a plot simply represents the normal pattern of variation in the level of expression, and the greater the slope the more variable the level of expression. It is not ever expected that an unusual level of expression in comparison with normal will be expected in all examples of a given type of cancer. That is the reason we look for such subgrouping of expression levels. JMJD2c shows a possible small subgroup of less than 5%, with one line expressing at a substantially higher level than all the others. The same may be true for EZH2, though here the results are less convincing as the higher levels of expression in the lines overlap those found in some normal samples. For the other genes there may be one or two lines with unusually high levels of expression that may be worth exploring.

These data, however, do not suggest that the 7 EPIDIACAN genes, with the possible exception of PRK1, are themselves candidates for genes whose expression change has been selected for, at least during colorectal cancer progression. This places the emphasis on looking at their downstream targets using the cell lines, taking into account the observed different levels of expression in different cell lines. Once this is done on a small subset of the lines, then the levels of expression of potentially interesting candidate genes can be further analysed, again

looking for clearly defined subgroups with different levels of expression.

Previous studies in our laboratory have suggested that EzH2 levels are associated with CSCs in our cultures, and that EzH2 was down regulated when the CSCs differentiated. This has now been confirmed by showing that knocking down EzH2 activity using siRNA increases CSC differentiation, as measured both by lumen formation and expression of the columnar cell differentiation marker Cytokeratin 20.

We previously gave preliminary evidence that F-actin (filamentous actin) is a useful marker of colorectal 1 (CRC) cell line derived lumens. We have confirmed the in vivo relevance of this by staining normal human colon cryosections with fluorescent phalloidin, which is specific for F-actin. In normal colon, F-actin is widely expressed in epithelial plasma membranes but is intensely enriched at the apical surface of enterocytes lining the crypt, corresponding to the brush border. This enrichment for F-actin is most probably due to the presence of microvilli on the colonic enterocytes. The intense staining of the muscularismucosum muscle layer was visible under the crypts. We next examined F-actin labelling in single cell derived colonies from a panel of six colon cancer cell lines, all grown under the same conditions in a three dimensional Matrigel matrix. In SW1222, confocal Z-sectioning demonstrated intense F-actin stress fibres were present on the apical cell membranes facing into the lumen. Three of the cell lines, LS180, SW1222, and C80 form colonies consisting of polarised cells surrounding central lumens visible by light microscopy. In fluorescence mode all phase visible lumens were clearly labeled by intense F-actin staining, although weaker F-actin labelling was also present in cell membranes. Generally more F-actin foci were visible than lumens observed by phase contrast alone, but in all cases smaller lumens were surrounded by polarised cells, indicating they were indeed just small lumens. The three other cell lines, HT29, HCT116 and DLD, did not form observable lumen colonies using phase contrast, and did not exhibit organised bright foci of F-actin staining, although variable expression of F-actin was visible in plasma membranes.

Because F-actin was enriched in lumens we examined whether other intestinal brush border markers were also present. Villin, an F-actin interacting protein associated with intestinal differentiation, was strongly enriched in lumens and its staining overlapped that for F-actin. The non-lumen forming cell lines HCT116 and DLD1 did not express Villin. This may in part be explained by their lack of expression of CDX1. Notably for HT29, villin was strongly and generally expressed but was not polarised and its staining did not overlap that for F-actin. Ezrin, another F-actin interacting protein enriched in the brush border, was also strongly enriched in lumens where it was clearly located at the apical cell membrane. Ezrin was only weakly present at non-apical plasma membranes and was often present as cytoplasmic specks. Interestingly, ezrin was strongly expressed in HCT116 but in a non-polarised manner indicating a failure to polarise this protein correctly.

We next examined whether formation of F-actin enriched lumens in colonies derived from a single cell precluded the presence of secretory lineages by co-staining of SW1222 colonies with phalloidin and anti-MUC2, a goblet cell specific mucin. Goblet cells were present in F-actin colonies grown from single cells, although their apical membranes appeared markedly smaller than those of the surrounding enterocyte cells. Thus single stem cells can differentiate along at least two lineages in lumen colonies. We

also examined expression of carcinoembryonic antigen (CEA), a membrane associated and secreted glycoprotein commonly used as a cancer marker. CEA was present almost exclusively in the lumens in C80 and SW1222.

#### **WP5 Clinical validation of selected epigenetic markers**

To determine if lumen formation or lack thereof had relevance to tumours in vivo, we first examined actin and ezrin polarisation in murine xenografts derived from the injection of 500 cells of HT29, HCT116 or SW1222 into the flanks of NOD/SCID mice. Mice were sacrificed after one month and resulting tumours removed and processed for FFPE tissue sections which were stained by hematoxylin and eosin or immunolabelled with anti-actin/ezrin. Hematoxylin and eosin staining of these xenograft tumours demonstrated poorly differentiated high grade tumour morphology for HCT116 and HT29 tumours that lacked lumens or actin/ezrin polarisation. SW1222 tumours exhibited a well differentiated phenotype with numerous lumens surrounded by polarised cells. Immunolabelling using antibodies to actin and ezrin showed that neither HT29 nor HCT116 exhibited polarisation of these markers whereas SW1222 showed marked polarisation, specifically in the lumens, thus indicating that the mouse stroma was able to induce polarisation and enterocyte differentiation of the growing tumour mass. Interestingly ezrin and actin were slightly spatially separated, unlike the Matrigel grown equivalents, suggesting elongated microvilli. Thus in vivo, cell lines grow in a manner similar to in vitro growth in Matrigel.

Because similar glandular structures, known as 'neoplastic glands' are a frequent feature of human colorectal adenocarcinomas, and indeed are routinely used by pathologists to grade tumours, we examined if these structures were labelled with the same markers as in vitro grown cell line derived lumens. We compared two colorectal tumours that had been graded previously by a qualified pathologist as either moderately differentiated (low grade) or poorly differentiated (high grade). Hematoxylin and eosin staining of tissue sections from the low- grade tumour confirmed the presence of numerous neoplastic glands, which were absent in the poorly differentiated tumour. Immunolabelling of sections from the same tumours indicated that, as with the xenografts, lumens in the low grade tumour were composed of polarised cells expressing actin and ezrin on the apical membranes, whereas no polarisation of these markers was observed in the poorly differentiated tumour. Note that typical of tumours, cellular debris was present in the lumen structure. Neoplastic lumen glands were also strongly positive for polarised membrane associated and secreted CEA, found almost entirely within the lumens in the moderately differentiated tumour, whereas CEA was non-polarised and appeared to be more membrane associated in the poorly differentiated tumour. Thus we have shown that at least three markers of in vitro lumens also mark in vivo glands. This, coupled with the remarkable polarised morphology of both types of structure, indicates that lumens from cell lines are virtually identical in nature to lumens in primary tumours.

As part of the evaluation of the possible significance of LSD1 gene expression in CRC we have tested the sensitive of a small representative panel of our CRC lines to LEAD -1, a specific inhibitor of the LSD-1 enzyme. None of the cell lines were killed or inhibited in their growth by LEAD-1 over a very wide range of drug concentrations. We have also shown that LEAD-1 does not affect cellular differentiation. These results

suggest that LSD-1 would not be a good target for further drug development in colon cancer.

#### **WP6 Development of epigenomic biomarker tests for diagnosis prognosis and therapy prediction**

We have continued our evaluation of variations in the procedures for handling patient blood samples for CTC detection. We are particularly concerned to assess the best approaches for both short and long term storage for freshly collected blood samples and to reassess the comparison between filtration and Ficoll-Hypaque partial purification of epithelial cells from the blood. We have also been testing various procedures for multiple antibody staining using our routine monoclonal antibodies, AUA1, an anti EpCAM and CAM5.2, against cytokeratins7/8 , together with other antibodies , for example against the differentiation control marker CDX1 and the prostate specific markers PSA and PSMA, as well as FISH for the common translocations found in prostate cancers.

We have continued to collect blood samples from CRC patients with a view to future evaluation of presence of Circulating Tumour Cells (CTCs) in relation to clinical outcome. Own main work under this heading, as a background to CTC characterisation from CRCs has been to develop a new approach to establishing primary cultures from fresh CRC patient tumour material. First of all we devised a novel mechanical disruption approach for the samples, which involves agitating samples with glass beads under controlled conditions. This leads to break up of the tumour material, but importantly crypt like structures ('neoplastic glands') are well maintained. These structures are filtered from single cells and larger debris and plated under non-adherent conditions in complex medium with growth promoting peptides. After several hours, well-formed spheroids can be observed amongst necrotic material as well cystic 'bubble' cells and debris which are frequently produced in primary cultures. Primary cultures switched to standard serum medium in adherent culture conditions attach and form colonies of monolayer cells. When embedded in matrigel, these spheroids form crypt like structures that grew over a period of months - 1, 2 and 3 months respectively.

Thus in summary, we conclude that lumen formation represents the same process in vivo as it does in vitro, and can therefore be used to identify stem cell populations in primary tumour derived cultures.

We confirmed that primary spheroids were composed of pure epithelial cells by staining for the epithelial specific markers CAM5.2 and CDX2, and the proliferation marker Ki67 confirmed that the cells were replicating. We have also shown that goblet cells are detected only in primary cultures derived from goblet cell expressing tumours. We repeated our procedure for a total of 31 tumours and found a very high success rate for short-term cultures, and a high success rate for long term cultures.

Our results show that single stem cells from colorectal cancer lines, when grown under 3D conditions, can differentiate into polarised structures with remarkable structural similarity to normal intestinal crypts. Lumen colonies grown from single cancer cells express several features of normal intestinal brush borders, including microvilli structural proteins (polarised F-actin, villin, ezrin), enzymatic activity (polarised DPPIV), and cell surface and secreted glycoprotein (CEA). Thus colorectal cancer stem cells that produce lumen colonies can

be considered to be differentiating predominantly along the enterocyte lineage, which is also the most common cell type of the normal colon. Nevertheless goblet cells, part of the secretory lineage, were also often present in colonies with F-actin lumens that had been grown from single cells, illustrating the multi-potent nature of these cancer stem cells. Importantly, we also show that several of these markers of in vitro lumen brush border formation are also expressed in apolarised manner by neoplastic glands present in primary tumours, which are similarly composed of polarised cells surrounding a lumen. Thus it is most likely that these neoplastic lumens also indicate stem cell differentiation in the same way as their in vitro counterparts. In support of this we found that a high proportion of primary tumour spheroid cultures derived from moderately/well differentiated tumours, which are known to be enriched for stem cells can form polarised lumen colonies when transferred into Matrigel. This shows how clearly different patterns of differentiation in primary tumours can be defined by these techniques. This definition of differentiation enlarges on the classical histopathologist's definition of differentiation and may be important for refining the definition of clinical prognosis.

**Participant 3. Carmen Jeronimo, IPOPFPG.**

**The major achievements are as follows: In summary**

Regarding prostate cancer: 1-We have explored the role of HMTs altered expression in PCa onset and progression and from the 37 genes explored we found that SMYD3 plays an important role in prostate carcinogenesis and therefore it may be useful as a therapeutic target in aggressive human PCa. 2- We did not find prognostic value in PCa patients for EZH2 and H3K18Ac immunoexpression in biopsies, contrarily to what has been previously reported.

Concerning colorectal tumors: 1-Lower LSD1 expression was observed in advanced stages of disease, suggesting that LSD1 may be associated with less aggressive CRCs. Conversely, higher H3K9me3 expression was observed in CRCs with regional lymph node metastases, which appears to be associated with the putative role of EZH2 in the progression of CRC. Likewise, higher H3K27me3 expression was observed in more invasive CRCs, CRCs with regional lymph node metastases and in CRCs at advanced stages of disease, as well as in CRCs with lymph and venous vessel invasion. These findings might be related with the putative role of EZH2 in CRC progression. 2- Concerning response to treatment, higher survival rates in patients with CRCs treated only with surgery were found for tumours displaying lower SETDB1 expression, suggesting that whenever the expression of SETDB1 occurs, the surgical treatment may not be enough to prolong the patient's survival. Importantly, LSD1 expression and the histone mark H3K9me3 may predict the response of CRC patients treated with Folfiri, and H3K9me3 and H3K27me3 expression may predict CRC patients' response to 5-FU/Leucovorine.

**In more detail:**

**WP3. Identification of downstream targets of epigenetic modifiers and proof of their role in prostate and colorectal cancer models.**

**Deliverables 3.2 (Month 24) have been achieved.**

We have developed engineered prostate cancer cell lines lacking SMYD3 in order to characterize the biological function of SMYD3. (WP3; Deliverable 3.2) To clarify the role of HMTs altered expression in PCa onset and progression and to translate those findings into clinically useful tools for management of PCa patients. To achieve this goal, epigenetic gene

expression of 37 HMTs was investigated by analysis of TaqMan® Arrays Plates in 10 primary PCa and 5 Morphological Normal Prostate Tissue (MNPT). This data was further confirmed in a larger and independent series of 150 primary PCa and 15 MNPT, and their mRNA expression levels were correlated with clinicopathological data.

We found that SMYD3, an H3K4 methyltransferase, was significantly overexpressed in tumor samples mainly in advanced stage disease (pT3b). Moreover, we stably silenced SMYD3 in LNCaP cell line and assessed the effects in cell viability, apoptosis and migration through standard assays. SMYD3 knockdown did not affect global H3K4 methylation levels but appears to impact in selective promoter regions. Although the absence of SMYD3 did not alter cell viability, it led to a significant decrease of PCa cells migration rate, as well as to an increase in apoptosis. Although additional studies in other PCa cell lines are required, our findings suggest that SMYD3 plays an important role in prostate carcinogenesis and therefore it may constitute a promising therapeutic target in aggressive human PCa.

**WP4.** Work performed for Deliverable D4.1 and 4.2 (scheduled for Month 24) and D4.3 (scheduled for Month 36). Collect and store biological samples of prostate cancer patients and controls, as well as relevant clinical data. From month one to 36th and after informed consent of the patients we were able to collect 240 fresh-frozen samples of prostate cancer tissue, as well as matched bodily fluids (urine and peripheral blood), which have been stored at -80°C. Simultaneously, bodily fluids from 200 healthy donors and patients harboring other urological pathologies have also been collected and stored for further analysis.

**WP5.** (cont.) The parts of work corresponding to Deliverables 5.2 (scheduled for Month 30), 5.3 (scheduled for Month 30), 5.4 (Scheduled for Month 30) and 5.5 (Scheduled for Month 30) are being pursued and have not been achieved yet. Work performed for Deliverable D5.2 (scheduled for Month 30). Establish the clinical value of factors including JMJD2C, LSD1, and PRK1 as a predictive parameter for aggressive and hormone-refractory prostate cancers. (Delivery month 30)

Deliverable D5.2 (scheduled for Month 30) is being pursued and has not been achieved yet.

Evaluation of clinical significance of EZH2 and global H3K18Ac in prostate cancer biopsies. Expression of EZH2 and H3K18Ac assessed by immunohistochemistry was evaluated in a series of 164 prostate biopsies from PCa patients and further correlated with standard clinical and pathological parameters. An integrated score was calculated for both assays and an empiric cutoff was established. Contrarily to what has been reported for prostatectomy specimens, EZH2 and H3K18Ac immunoexpression in PCa at diagnosis did not show any prognostic value although EZH2 immunoexpression correlated with higher serum PSA and lower Gleason score.

Work performed for Deliverable D5.5 (scheduled for Month 30). Establish the clinical value of factors including Usp22, SMYD3, Ezh2 and JMJD3 in colon cancer tissues/biopsies (Delivery month 30).

We characterized the expression of four histone modifying enzymes, including the histone methyltransferases EZH2, SMYD3 and SETDB1 and the histone demethylase LSD1, as well as the respective histone marks H3K9me3

and H3K27me3 by immunohistochemical assays in a series of 98 colon cancer(CRC) patients, clinically and pathologically well characterized. The evaluation of SMYD3 expression was considered positive if microscopic staining was present in greater than 30% of the tumor cells. The remaining enzymes and both histone marks were assessed using a modified HistoScore (H-score).

Histone methyltransferases EZH2 and SMYD3 and the histone demethylase LSD1 were overexpressed in CRC cells, suggesting a role for these enzymes in the neoplastic transformation. Interestingly, the expression of SMYD3 was detected in the cell membrane and cytoplasm of CRC cells, in contrast with the nuclear expression observed in the remaining histone modifying enzymes. In addition, the expression of histone marks H3K9me3 and H3K27me3 was increased in CRC cells, consistent with the increased expression of the abovementioned histone modifying enzymes.

Differences in the expression of the selected enzymes or histone marks in the nuclei of tumor cells and their expression in the normal mucosa. The expression of histone modifying enzymes and the expression of histone marks were associated with several clinicopathological features. Higher EZH2 expression was observed in more invasive CRCs and CRCs with regional lymph node metastases, suggesting that EZH2 may have a role in tumor growth and cell invasion. Higher SETDB1 expression was observed in left-sided CRCs, suggesting a putative role in the carcinogenesis of CRCs with chromosomal instability.

Lower LSD1 expression was observed in more invasive CRCs and in CRCs at advanced stages of disease, suggesting that LSD1 may be associated with less aggressive CRCs. Higher H3K9me3 expression was observed in CRCs with regional lymph node metastases, which appears to be associated with the putative role of EZH2 in the progression of CRC. Higher H3K27me3 expression was observed in more invasive CRCs, CRCs with regional lymph node metastases and in CRCs at advanced stages of disease, as well as in CRCs with lymph and venous vessel invasion, which appears to be associated with the putative role of EZH2 in the progression of CRC.

Regarding the response to treatment we observed higher survival rates in patients with CRCs treated only with surgery disclosing lower SETDB1 expression, suggesting that whenever the expression of SETDB1 occurs, the surgical treatment may not be enough to prolong the patient's survival. Higher survival rates in patients with CRC treated with Folfiri were associated with high LSD1 and H3K9me3 expression in cancer cells, suggesting that LSD1 expression and the histone mark H3K9me3 can predict the response to this treatment modality. Higher survival rates of patients with CRC treated with 5-FU/Leucovorine were associated with high H3K9me3 and H3K27me3 expression in cancer cells, suggesting that these patterns of methylation can predict the response to this treatment modality.

Univariate analyses of the association between the expression of the selected enzymes or histone marks in CRC with different therapeutics and respective overall survival. Finally, we verified that the evaluation of histone modifying enzymes and histone marks using a low/high expression system reproduced the results obtained with the H-score, constituting an easier system to evaluate the expression of the histone modifying enzymes and histone marks in the routine of a pathologist. This work accomplished the identification of new biomarkers that can be used in clinical

practice and gives rise to new directions in the study of CRC carcinogenesis.

#### **WP6. Development of epigenomic biomarker tests for diagnosis prognosis and therapy prediction**

The parts of work corresponding to Deliverables 6.1 (scheduled for Month 36) and 6.2 (scheduled for Month 36) are being pursued and have not been achieved yet. Work performed for Deliverable D6.2 (scheduled for Month 36). Evaluation of epigenetic markers in cancer cells from body fluids (Delivery Month 36). We have identified several epigenetic markers for prostate cancer in WP5. However, we were unable to detect these surrogate markers either at RNA or protein level in human body fluids such as urine.

#### **Participant 4. Laszlo Tora, GIE-CERBM.**

S&T results on USP22 and the deubiquitination module from GIE-CERBM. The tightly controlled deubiquitination activity of the human SAGA complex differentially modifies distinct gene regulatory elements. The multisubunit SAGA (Spt-Ada-Gcn5 acetyltransferase) coactivator complex facilitates access of general transcription factors to DNA through histone acetylation mediated by GCN5. USP22 (ubiquitin-specific protease 22) was recently described as a subunit of the human SAGA complex that removes ubiquitin from monoubiquitinated histone H2B and H2A in vitro. We described an allosteric regulation of USP22 through multiple interactions with different domains of other subunits of the SAGA deubiquitination module (ATXN7, ATXN7L3, and ENY2). Downregulation of ATXN7L3 by short hairpin RNA (shRNA) specifically inactivated the SAGA deubiquitination activity, leading to a strong increase of global H2B ubiquitination and a moderate increase of H2A ubiquitination. Thus, SAGA is the major H2Bub deubiquitinase in human cells, and this activity cannot be fully compensated by other deubiquitinases. Here we show that the deubiquitination activity of SAGA is required for full activation of SAGA-dependent inducible genes. Interestingly, the reduction of the SAGA deubiquitination activity and the parallel increase in H2B ubiquitination at inducible target genes before activation do not induce aberrant gene expression. Our data together indicate that different dynamic equilibria of H2B ubiquitination/deubiquitination are established at different gene regulatory elements and that H2B ubiquitination changes are necessary but not sufficient to trigger parallel activation of gene expression.

The structural plasticity of two different SCA7 domains of the SAGA deubiquitination module defines their differential nucleosome-binding properties.

SAGA a coactivator complex is involved in chromatin remodelling, and harbours both histone acetylation and deubiquitination activities. Human (h) ATXN7 (homologue of yeast Sgf73) and hATXN7L3, two subunits of the human SAGA deubiquitination module, contain an SCA7 domain characterized by an atypical zinc-finger. We show that the yeast Sgf73-SCA7 domain is not required to recruit Sgf73 into SAGA. Instead, it binds to nucleosomes, a property that is conserved in the human ATXN7-SCA7 domain, but is lost in the hATXN7L3 domain. The solution structures of the SCA7 domain of both hATXN7 and hATXN7L3 reveal a new, common zinc-finger motif at the heart of two distinct folds, providing a molecular basis for the observed functional differences.

#### **Role for H2Bub in DNA repair and carcinogenesis**

While enzyme-mediated epigenetic control of gene transcription is a critical aspect of embryonic development and cellular differentiation, this same mechanism is often deregulated in human diseases, where aberrant gene expression or repression is a hallmark of cancer and other diseases. There is growing evidence that amplification, mutation and other alterations of epigenetic enzymes are molecular causes of certain cancers and several human diseases. A number of observations suggest that deregulation of histone H2B ubiquitination and the resulting effect on transcription may be key events in cellular transformation and metastasis. First, gene expression analysis of RNF20 depleted cells suggested that RNF20 acts as a putative tumor suppressor gene. Indeed, the expression of several proto-oncogenes and proliferation-related genes was increased after reduction of H2Bub via RNF20 depletion. In contrast RNF20 positively regulates p53 and different studies showed that RNF20 and WAC regulate p53-dependent genes in response to DNA damage. Finally, RNF20 was found to be silenced by increased methylation of the RNF20 promoter in breast cancers. Interestingly, transcriptional profiling of tumor cells identified USP22 that catalyzes the opposite enzymatic reaction, as part of an 11-gene signature associated with poor prognosis. The presence within this signature of several genes with known oncogenic properties such as BMI1 a PRC1 subunit that is overexpressed in a variety of cancers further support that deregulation of H2Bub might be involved in cancer development. In support of a role for H2Bub in cellular transformation, two recent studies have implicated this mark in repair of DNA double strand breaks.

Indeed the RNF20/RNF40 complex was shown to be recruited at DNA damage sites and it is proposed that the consequent H2Bub would allow chromatin fiber decompaction and timely recruitment of repair proteins. The role of H2Bub in both transcription elongation and DNA repair could suggest a unifying mechanism based on local chromatin relaxation. However, contradicting results were reported regarding the influence of H2Bub on chromatin structure. Although in vitro studies using fully ubiquitinated chromatin templates demonstrated that H2B ubiquitination is sufficient to interfere with compaction of the 30 nm chromatin fiber, in vivo MNase sensitivity studies suggested that absence of H2Bub opens the chromatin, whereas increase of H2Bub would make the chromatin more compact.

Altogether these observations further suggest that deregulation of H2B ubiquitination may contribute to cancer development. Thus, epigenetic enzymes such as the histone deubiquitinating enzymes have become attractive targets for drug discovery, and specific and sensitive assays are needed for screening and characterizing potential inhibitors of these enzymes. The development of inhibitors of the different enzymes involved in the regulation of H2Bub will help to shed new light on the various functions of histone H2B ubiquitination and might provide new avenues for therapeutic intervention.

(WP1; Deliverables D1.1, D1.2) (GIE-CERBM). We have generated ten different rabbit polyclonal and two different mouse monoclonal antibodies against human USP22. These antibodies were raised against either synthesized USP22 peptides that were coupled to carrier proteins, or against bacterially overexpressed and purified USP22 fragments.

Concerning the mouse monoclonal antibodies: supernatants from hybridoma cell clones were tested by ELISA detection using recombinant proteins or peptides as antigens. Positive clones were subcloned several times, retested by ELISA and western blotting. The subclones selected were

expanded and used for injection of mice to produce high concentrations of antibodies in ascites fluid. Using this procedure we obtained two slightly positive mAbs, which recognize weakly the endogenous human USP22.

Concerning the rabbit polyclonal antibodies: New Zealand White rabbits were immunized by a standard, 70-day immunization program. Bleeds were collected and assayed in western blots of cellular extracts. The antibodies were then purified by affinity chromatography and their performance in western blots assays were tested using recombinant proteins or purified endogenous human SAGA complexes of which USP22 is a subunit. The best performing antibodies were selected and further evaluated for their ability to work in ChIP assays and immunohistological applications. In summary, we met deliverables 1.1 and 1.2 in full and delivered antibodies against human and mouse USP22 to the consortium.

(WP2; Deliverable 2.1) USP22 cell models (GIE-CERBM)  
Eukaryotic expression vectors (in pCDNA3-puro) have been generated to overexpress hUSP22 with a Flag or a HA tag. These vectors have been tested in transfection experiments for their efficiency. We have also generated an expression vector expressing a catalytically dead form of USP22. Four different shRNA silencing vectors (in pSUPER-puro) have also been generated to knockdown the expression of USP22. Their efficiency has been tested. Moreover, several of this shRNA sequences have been transferred in a lentiviral transfection system and we obtained several clones of stable knockdown cell lines.

(WP2, Deliverable 2.3) Development of Usp22 and Atxn7l3 (a key allosteric regulatory factor of USP22 enzymatic activity) animal models (GIE-CERBM)

Through the European consortium EUCOMM (see <http://www.knockoutmouse.org/about/eucomm> online), we have received the knockout (KO) ES cells for Usp22 and Atxn7l3. We got Usp22 conditional KO ES cells which allow the generation of different types of KO:

- Constitutive clean mutants, for which part of the gene has been replaced by a LoxP site;
- Floxed mutants allowing tissues specific mutation;
- Knock-in of a LacZ reporter gene under the control of targeted gene's promoter allowing the analysis of its expression profile.

Usp22 KO ES cells passed the quality control performed by the Mouse Clinical Institute (MCI) and have been injected to blastocysts in order to generate KO mice. Germ line transmission of the mutated allele from chimeric mice was obtained and heterozygotes were crossed with mice expressing the Flp recombinase to delete the Neo cassette. Clean heterozygote animals were bred to obtain the homozygote animals.

EUCOMM has only generated non-conditional Atxn7l3 KO. The Atxn7l3 chimeric mice have been generated by the MCI and germ line transmission has been confirmed recently. Heterozygotes were bred, and thus, we generated Atxn7l3-null mice.

Participant 5: Iannis Talianidis, BSRC Al. Fleming

The activities of the group in this program were:

1. Generation of antibodies against SMYD3.
2. Validation of SMYD3 as biomarker in human colon cancer biopsies.
3. Generation and analysis of animal models lacking Smyd3 and overexpressing Smyd3 in intestine.
- 4.

Generation and analysis of experimentally induced colon cancer. 5. Global expression profiling and occupancy analysis of Smyd3-regulated genes. 6. Provide experimental samples from animal models to the partners of the network.

#### **1. Generation of antibodies against SMYD3. (WP1; Deliverable D1.1)**

Initially we have tested several commercially available Smyd3 antibodies (Abcam, Santa Cruz, Sigma) using western blots from liver extracts of wild type and Smyd3 transgenic (Smyd3-Tg) animals and by immunohistochemistry in frozen or paraffin-embedded liver sections. The antibodies did not perform well in any of the applications. Next we generated a polyclonal antibody by immunizing 2 rabbits with a KLH-conjugated N-terminal peptide of Smyd3. The collected sera recognized recombinant Smyd3 as well as overexpressed Smyd3 in extracts prepared from CMV-Smyd3 transfected cells. The peptide antibodies did not recognize endogenous Smyd3. After this we immunized rabbits with recombinant full-length Smyd3 protein. The sera of these mice were tested in different applications: They gave a good signal in western blots of extracts containing overexpressed proteins. The antibody also gave good result in detecting endogenous Smyd3 by western blots and immunoprecipitations.

Very good signals were detected in Smyd3 transgenic mice and transfected cells in western blot and immunocytochemistry applications. Satisfactory results were obtained in detection the endogenous protein. Deliverable D1.1 has been achieved.

#### **2. Validation of SMYD3 as biomarker in human colon cancer biopsies. (WP3, WP5; Deliverable 3.6 and 5.5)**

The antibody was extensively tested in immunohistochemistry applications in mouse colon cancer sections and human colon cancer. Parallel measurements of RNA levels by RT-PCR were performed. The histochemical signals correlated well with the expression of Smyd3. Deliverables 3.6 and 5.5 has been achieved.

#### **3. Generation and analysis of animal models lacking Smyd3 and overexpressing Smyd3 in intestine. (WP2; Deliverable 2.3)**

#### **4. Generation and analysis of experimentally induced colon cancer. (WP2; Deliverable 2.5).**

To generate Smyd3 KO mice we used a gene-trap ES cell clone to inject mouse blastocysts. Chimeric mice and germ-line transmission was obtained before the initiation of the program. During the reporting period we obtained a good number of homozygous mice to perform experimental work. The mice look overall normal. Histological analysis of livers and intestines did not show major morphological alterations. We have performed drug-induced liver and colon cancer induction programs using DEN only and DEN-TC (10 and 7 months treatment, respectively) and DMH (3 months treatment) protocol in 2 groups of mice. Each group consists of 10 animals.

a. To induce colon cancer, 2 months old mice were injected with DMH, followed by treatment with DSS in drinking water for 1 week. The mice were then left for 2 weeks with normal drinking water. The DSS treatment was repeated two more times and the mice were sacrificed. Smyd3 KO mice exhibited inflammatory response similar to wild type mice, but essentially no formation of adenomas.

b. To induce liver cancer, at day 14 after birth each litter received a single intraperitoneal injection of the DNA-damaging tumor initiator diethylnitrosamine (DEN) and analyzed at 10 months age (DEN only protocol). In DEN-TC protocol, two weeks after DEN treatment, and every two weeks afterwards, the animals were given intraperitoneal injection of the hepatotoxic nuclear receptor CAR ligand TCPOBOP (TC) for 4 to 6 months. Using this protocol full-blown liver tumor was detectable at 4 months after treatment in wild type mice.

Using a newly developed antibody against Smyd3, we found that Smyd3 protein is upregulated in colon cancer. Double staining with Ki67 revealed that in proliferating cells Smyd3 is mainly localized in the nucleus. Particularly interesting is the finding showing the lack of increase of Ki67 positive cells in Smyd3 KO mice and the lack of loss of E-cadherin staining in these cells, suggesting that Smyd3 plays role in carcinogenesis mainly through promotion of epithelial to mesenchymal transition (EMT). This is probably achieved by the induction of matrix metalloproteases, which we have identified as bona fide targets of Smyd3 (see below). The number of proliferating cells in the crypts was not affected by Smyd3 deletion.

Importantly, our results suggested that in colon cancer Smyd3 does not act through transcriptional activation of S-phase specific genes, but rather through promoting EMT.

Ki67 (Red) and E-cadherin (Green) staining of colon sections. Blue: DAPI staining of nuclei. Note the loss of E-cadherin staining from the membranes of WT-treated cells.

Smyd3 deficiency also protected mice against hepatocarcinogenesis. DEN-TC treatment resulted in much less tumor foci in Smyd3 KO mice compared to WT mice, while hepatomegaly was not affected. Similar results were obtained with the DEN-only protocol, which induces HCC without concomitant hepatomegaly.

10 months Livers from DEN-only treated mice.

Further studies revealed that Smyd3 inactivation did not affect DEN-induced cell death, inflammation or fibrosis. In addition the proliferation promoting effect of TCPOBOP (TC) was also detected in Smyd3 KO mice. We also performed partial hepatectomy, to study the effect of Smyd3 on liver regeneration. Interestingly, Smyd3 KO mice exhibited similar to wild type regenerative capacity. Taken together, these data suggested that Smyd3 does not affect "normal" cell proliferation. Its pro-carcinogenic function is rather elicited by activation of MMPs and subsequent promotion of EMT.

#### **Smyd3 Tg mice:**

Smyd3 Tg mice were generated using a construct containing the full-length Smyd3 cDNA under the control of the liver-specific TTR promoter/enhancer. The transgenic animals express 5'HA and 3'Flag tagged version of full-length Smyd3, specifically in hepatocytes. We have expanded 2 transgenic lines, where transgene expression was monitored by RT-PCR and western blot analysis. HA-tag antibody detected a 52 kD band only in whole cell extracts, while Flag antibody detected a 52 and 48 kD band in whole cell extracts and a 48 kD band in nuclear extracts. This suggests that in the nuclear form of Smyd3 the N-terminal region is cleaved.

Smyd3 Tg mice did not develop tumors spontaneously (at least within 10 months of age) but, as expected, somewhat accelerated DEN-induced carcinogenesis.

**Deliverables 2.3 and 2.5 have been achieved.**

**5. Global expression profiling and occupancy analysis of Smyd3-regulated genes.**

**(WP 3, Deliverable 3.3)**

We attempted to identify genome wide occupancy patterns of Smyd3 using several antibodies (including those developed by us) recognizing endogenous Smyd3. None of them worked in ChIP assays. As an alternative, we took advantage of our transgenic mice, where the nuclear form of Smyd3 can be detected by Flag antibodies. ChIP-seq analysis revealed several (more than 8000) locations of the genome bound by Smyd3. About 20% of them were located in promoters. While several interesting target genes were identified, it was interesting to note that Smyd3 was not recruited into the regulatory regions of S-phase specific genes. As mentioned above, target genes most relevant to the carcinogenesis process were those regulating EMT. These include members of the matrix metalloprotease family, which have also been detected in human colon cancer. Chromosome-wide (chr12) distribution of peaks in Smyd3 ChIP-seq experiment. 2 biological replicates show similar binding pattern. Deliverable 3.3 has been achieved.

**6. Provide experimental samples from animal models to the partners of the network.**

Several exchanges of scientific materials between EPIDIACAN members have been realized during the project.

**7. Other activities**

Another line of our research in this program was to identify the lysine specificity of Smyd3. Previous studies established that Smyd3 is a histone 3 lysine 4 methylase. We performed several in vitro studies using bacterially or baculovirus expressed Smyd3 recombinant proteins. While in the presence of HSP90 we could observe some methylation at H3K4, this activity was very low compared to other enzymes. On the other hand we identified Smyd3 as an H4K20 methylase, which seems to be the main specificity of the enzyme. We have also been searching for novel, non-histone substrates of Smyd3. We made use of our transgenic mice to perform immunoprecipitations followed by quantitative mass-spectrometry. This effort led to the identification of several cytoplasmic proteins including Cyld. Although, Cyld methylations still needs verification, we hypothesized that the mechanism by which Smyd3 modulates hepatocarcinogenesis may involve regulation of NFkB signalling. While the roles of the components of NFkB signalling in cancer have been studied before, no information about the potential role of the deubiquitinase Cyld was available. To this end we generated mice that specifically lack Cyld in the liver. To our surprise, we observed that Cyld-deficient mice develop spontaneous hepatocellular carcinoma. Detailed description of our results can be found in the Cancer Cell publication indicated in S4. Briefly, we showed that liver-specific disruption of Cyld triggers hepatocyte cell death in the periportal area. This effect was elicited via spontaneous and chronic activation of TAK1 and JNK. Hepatocyte death was followed by hepatic stellate cell and Kupffer cell activation, which promoted progressive fibrosis, inflammation, TNF production and expansion of hepatocyte apoptosis towards the central veins. At later stages, compensatory proliferation resulted in the spontaneous development of cancer foci with features of re-expression of oncofetal hepatic and stem

cell-specific genes. These results established the important role of Cyld in regulating liver cancer.

### **Conclusion**

Our studies revealed an important role of Smyd3 in liver and intestinal cancer. The mechanistic basis of its function involves promotion of epithelial-mesenchymal transition. The possibility of regulating tumorigenic signalling pathways was also raised, which opens new avenues for future research. It was also shown that Smyd3 could serve as a good biomarker for colon cancer. Future studies may be directed towards developing and testing drugs specifically modulating the activities of the enzyme.

### **Participant 6: Roland Schuele, UKL-FR**

**WP1:** We generate several new antibodies with the goal of increased specificity for histological, ChIP and other applications. These include antibodies against JMJD2A-D, LSD1, and PRK1. For immunization purified recombinant proteins were used. For the production of polyclonal antibodies, New Zealand White rabbits were immunized by a standard, 70-day immunization program. Test bleeds were collected and assayed in western blots of cellular extracts and with purified recombinant target proteins. Then the antibodies were purified by affinity chromatography and their performance was verified in western blots, ChIP assays and immunohistological applications. In summary, we met deliverables 1.1 and 1.2 in full and deliver superior antibodies for JMJD2A-D, LSD1, and PRK1 to the consortium.

### **WP2: Cell culture models**

We established and optimized siRNA-mediated knock-down strategies to reduce the levels of JMJD2C, LSD1, and PRK1 in human prostate cancer cell lines such as LNCaP. for the purpose of generating cell lines with reduced. For inducible expression we developed the tetracycline-controlled approach to conditionally overexpress shRNAs using a lentiviral gene silencing system. For overexpression we also used lentiviral gene delivery system of the various wild type and mutant proteins. Bona fide expression of the corresponding gene was verified by RT-PCR and western blot analysis.

### **JMJD2c, LSD1, PRK1 KO, transgenic mice**

To investigate the biological and pathophysiological functions of JMJD2C, LSD1, and PRK1 and their contribution to the development of cancer we cloned targeting constructs that allow the Cre-loxP mediated deletion of PRK1 exon 1 of PRK1 and LSD1 respectively. We generated homozygous floxed animals (LSD1 and PRK1) and breeding colonies of LSD1 floxed animals are established while the breeding colony of PRK1 floxed animals is expanding. Next, we generated animals that have prostate specific gene knockout by breeding LSD1flox/flox animals with transgenic ARR2Probasin-Cre (PB-Cre4) and ARR2Probasin-CreERT (PB-CreERT) mice that express Cre recombinase specifically in the prostate epithelium in a constitutive or Tamoxifen inducible manner, respectively. In addition, we produce knockout mice for the demethylase JMJD2C by using gene-trap technology. We initiated the analysis of the prostate-specific knockout animals by histological and biochemical means and functional tests assaying for altered performance and physiology.

Furthermore, to understand if JMJD2C, LSD1, or PRK1 can be causative in the development of prostate carcinomas and metastasis (drivers of

tumourigenesis) we created transgenic mice that express these genes under the control of the probasin promoter specifically in prostate epithelium. The physiological impact of enforced expression of the demethylase JMJD2C, LSD1 or PRK1 on differentiation, proliferation, tumour development, and metastasis is being analysed by the experimental approaches described above. Also we initiated to use a synthetic biology approach to reconstruct the PRK1/LSD1/JMJD2C pathway by generating triple transgenic mice that show enforced expression of the three proteins in prostate epithelium. In summary, we met deliverables 2.1 and 2.3 in full and deliver KO and transgenic mice respectively, for JMJD2C, LSD1, and PRK1 to the consortium.

**WP3:** To characterize the biological function of the demethylases JMJD2C, LSD1 and the kinase PRK1 we combined a genome-wide occupancy analysis performed by ChIP-sequencing with global expression profiling data derived from Affimetrix platform, applied comparative analysis and retrieved JMJD2C, LSD1 target genes such as EGFR, CDK1 or the PRK1 target KLK2 and KLK3. The physiological role of the various target genes will be analysed in detail.

Secondly, we performed SILAC screens for proteins interacting with LSD1 and JMJD2C respectively, and characterize among members of the CoREST and NURD complex several novel interactors. The association of these novel proteins with LSD1 and JMJD2C was verified in vitro and in vivo. The analysis of the physiological role of the various interactors is ongoing.

Finally, we characterize by an unbiased genome wide screening strategy using, kinases that modify LSD1, and potentially also other members of the LSD1-demethylases associated complex. Phosphorylation was identified by mass-spectrometry, by the use of modification-specific antibodies, and verified by mutagenesis. The analysis of the consequence this phosphorylation step in respect to demethylation, complex assembly, target gene regulation and control of tumour cell proliferation and metastasis in vitro and in vivo is initiated and ongoing. In summary, we met deliverables 3.2 to 3.4 in full and deliver this data to the consortium.

**WP3: D 3.2:** To investigate the biological and pathophysiological functions of JMJD2C, LSD1, and PRK1 and their contribution to the development of cancer we generated homozygous floxed animals (LSD1 and PRK1). A breeding colony of PRK1 floxed animals is expanding. So far we have obtained three PRK1 null animals. The first physiological and histological analysis revealed no obvious phenotype of the PRK1 knockout animals. Further detailed analyses are ongoing. To characterize the functions of LSD1 we generated prostate specific gene knockout by breeding LSD1<sup>flox/flox</sup> animals with transgenic ARR2Probasin-Cre (PB-Cre4) mice that express Cre recombinase specifically in the prostate epithelium. The detailed biochemical and histological analysis revealed no obvious phenotype of the prostate specific LSD1 knockout animals. Further detailed analyses are ongoing. Next, we ubiquitously deleted LSD1 using a Rosa26-Cre deleter. Homozygous LSD1 knockout animals stop development at around E7.5-8.0 due to impaired trophoblast stem cell development. Furthermore, we produce knockout mice for the demethylase JMJD2C by using gene-trap technology. The detailed biochemical, histological, and functional assaying for altered performance and physiology revealed no obvious phenotype of the knockout animals. Further analyses are ongoing. In addition, to understand if JMJD2C, LSD1, or PRK1 can be causative in the development of prostate carcinomas and metastasis

(drivers of tumourigenesis) we created transgenic mice that express these genes under the control of the probasin promoter specifically in prostate epithelium. The physiological impact of enforced expression of the demethylase JMJD2C, LSD1 or PRK1 on differentiation, proliferation, tumour development, and metastasis was analysed as shown below. In summary, the data uncover that LSD1 is able to drive tumorigenesis (ADC: adenocarcinoma). Also we initiated to use a synthetic biology approach to reconstruct the PRK1/LSD1/JMJD2C pathway by generating double and triple transgenic mice that show enforced expression of the three proteins in prostate epithelium. The results demonstrate that /LSD1/JMJD2C double transgenic mice more robustly drive tumour development compared to the LSD1 transgenic mice. Importantly, in both mouse models tumours are only established late in life (between 45-90 weeks). The analysis of the triple transgenic PRK1/LSD1/JMJD2C mice is ongoing.

D 3.3: To identify LSD1 and PRK1 regulated genes we performed global analysis of gene expression by RNA-seq analysis. In PC3 cells we detected 488 and 1355 genes differentially regulated by LSD1 and PRK1, respectively. About half of the genes are either up- or downregulated. The bioinformatic analysis uncovered that LSD1 controls a gene set regulating transcription proliferation and metabolism. In contrast the PRK1 controlled gene set is associated with migration and invasion. In addition, we used the antibodies developed in WP1 to establish LSD1 ChIP-seq methodology. The genome-wide chromatin binding profile of LSD1 in LNCaP and PC3 cells was established. The bioinformatic analysis uncovered that LSD1 is enriched at promoters, defined by 2000bp around the transcription start site. LSD1 locations overlap with active histone marks such as H3K4me3 but not with the repressive mark H3K27ac. HOMER analysis identified AR, REST, and FOX binding motifs as significantly enriched motifs.

D 3.4: To identify LSD1 interacting proteins we used the antibodies developed in WP1 to immunoprecipitate endogenously expressed LSD1 in LNCaP and PC3 cells. The immunoprecipitated LSD1-associated proteins were identified by mass-spectrometry. Interestingly, we were able to identify the CoREST complex as interaction partner. However, we did not detect any member of the NuRD complex. Interestingly, we identified novel signalling molecules such as dynactin 1, dynactin 2, or protein phosphatase 12 as interactin partners. The functional analyses of these novel LSD1 associated proteins is ongoing.

D 3.5: We characterized by an unbiased genome wide screening strategy, kinases that modify LSD1 and analyzed the consequences of this modification on biological function. In vivo, LSD1 is modified by post-translational modifications such as acetylation and phosphorylation. We expressed and purified a pool of modifiers i.e. enzymatic active forms of all known acetylases and 180+ kinases covering a large percentage of all cytoplasmic and nuclear kinases present in the human genome. LSD1, expressed either in E. coli or insect cells, was tested for modification by the modifiers. Phosphorylation of LSD1 at position 119 was identified by mass-spectrometry. We developed phospho-specific antibodies (anti LSD1Tph119) and used the modification-specific antibodies to confirm the data obtained by mass-spectrometry. Phosphorylation was verified by mutagenesis (LSD1T119A). Our analyses reveal that the protein kinases CDK5 and PKA are able to phosphorylate LSD1 at position T119. Next, we characterized the consequence of this phosphorylation in respect to demethylation, complex assembly, target gene regulation and control of tumour cell proliferation and metastasis. Phosphorylation of LSD1 at

position T119 does not alter demethylation or complex assembly. Also, we did not detect alterations in target gene regulation. Next, we generated stable LNCaP cell overexpressing the mutant LSD1T119A. The characterization of tumour cell proliferation and metastasis of parental LNCaP and LNCaP overexpressing the mutant LSD1T119A in xenograft models is ongoing. In summary, we met the deliverables and deliver this data to the consortium.

**WP5:** D 5.2 and: D 5.2: To establish the clinical value of JMJD2C, LSD1, and PRK1 as a predictive marker of prostate cancer we initiated to characterized a study cohort of 500+ patients, which underwent radical prostatectomy for locally limited diseases within a single surgical center and were followed-up between 3 to 9 years. All prostate specimens were classified according to TNM stage and Gleason score following defined procedures and used to generate tissue microarrays (TMAs). Patients were be grouped into two categories according to the following tumour parameters: Group 1 (high-risk tumours): pT3 or higher, all pN0M0, Gleason score 8 or higher. Group 2 (low-risk tumours): pT2c or lower, all pN0M0, Gleason score 7 or lower. So far we have use the TMAs for immunostaining a panel of antigens such as LSD1 PRK1, p53, Ki67 etc.

Taken together, the data show that LSD1 is predictive marker of prostate cancer superior to PSA. Las1 levels clearly correlates with relapse free survival time. In summary, we met the deliverables and deliver this data to the consortium.

#### **Participant 7. Thomas Henkel, Targos.**

The cooperation with the Clinical Research Group (KF0179) at the University of Göttingen was continued to get access to tumor samples from two prospective rectal cancer trials in which patients have preoperatively been treated by chemo-radiotherapy (CRT). In the first trial (from 1995 to 2002) follow-up data covered at least five years (CAO/ARO/AIO-94), the second trial is still on-going (CAO/ARO/AIO-04). The trials were approved by the medical ethics committees of all participating hospitals.

Meanwhile ngreater than150 FFPE blocks of biopsies prior to neoadjuvant CRT and ngreater than60 FFPE blocks from resection specimens post-treatment have been collected. As controls, n=40 resection specimens of patients not undergoing neo-adjuvant treatment have been collected.

Whereas the main objective of the Clinical Research Group was to determine DNA stability and repair genes as predictive markers, our objective was to evaluate the predictive value of epigenomic markers.

In addition to the clinical samples from the prospective trials, multi-tissue micro-arrays from various tumor indications were generated from banked samples deriving from the tumor bank of the comprehensive cancer care center Cologne/Bonn (Prof. Büttner) and from commercial sources providing tissues with patient informed consent. The indications represented on the tissue micro-arrays include various stages of prostate cancer, in order to correlate expression of epigenomic markers with stage and grade of the tumor diseases. We added several other tumor indications such as NSCLC, lymphoma, breast cancer to broaden the search for interesting indications for the markers.

Antibodies generated in WP1 have been tested for potential use in IHC on the provided tissues. Only for LSD1 and PRK antibody robust IHC

protocols could be established. Neither LSD1 nor PRK showed any correlation to therapy or prognosis on the clinical CRC samples or on the banked samples. In addition, no prognostic value for either PRK1 or LSD1 beyond the published results in prostate cancer or neuroblastoma could be established in the tested tumor tissue types. LSD1 expression in prostate cancer samples correlates well with tumor grading, but is not an independent marker. Complementary to the candidate antibodies from WP1 some commercially available antibodies against PTEN, pHH3 and pERK were tested on the tissues.

**WP 4.1** In cooperation with the Clinical Research Group (KFO179) at the University of Göttingen tumor samples of two prospective rectal cancer trials were collected, in which patients have preoperatively been treated by chemo-radiotherapy (CRT). In the first trial (from 1995 to 2002) follow-up data covered at least five years (CAO/ARO/AIO-94), the second trial is still on-going (CAO/ARO/AIO-04). The trials were approved by the medical ethics committees of all participating hospitals.

Meanwhile ngreater than150 FFPE blocks of biopsies prior to neoadjuvant CRT and ngreater than60 FFPE blocks from resection specimens post-treatment have been collected. As controls, n=40 resection specimens of patients not undergoing neo-adjuvant treatment have been collected. The Targos pathologist not only served as reference pathologist to evaluate tissues, but he served as clinical coordinator to secure access to the tissue samples.

**WP 4.2** The planned SEAL trials, which was planned to be a prospective randomized phase III trial of limited versus extended lymph node dissection in patients with intermediate/high risk prostate cancer- (Trial number AP49/08) was finally not funded and thus was not started as planned in 2010. A second attempt for funding was not approved in 2011/2012. Thus no prospectively collected prostate cancer samples were available for analysis. These samples were replaced by retrospectively collected tumor bank samples (see WP 5.1) and commercially available tissues.

**WP 5.1** During the reported period Targos generated further multiple tissue micro-arrays (TMA) of high quality (Multiblock melting technology) from anonymized tumor bank samples including clinical data, mainly derived from the tumor bank of the comprehensive Cancer Care Center Cologne/Bonn (Prof. Büttner)

A total of 29 TMAs representing 1.362 tumor tissue samples in duplicate including pairs of primary tumors and metastases, was generated in the reported period from the following tumor indications: prostate, colon, breast, lung, kidney, stomach, head and neck, ovary, cervix, lymphoma and skin.

The TMAs were generated by selecting relevant FFPE tissue blocks from the tumor bank or commercial sources by a postdoctoral scientist, preparing H&E stained sections by a technician, indicating relevant areas by a pathologist, punching the relevant areas with a 6 mm core puncher from the blocks by a technician followed by the proprietary Multiblock assembly technology. The 60 core TMAs usually include two representative tumor cores per patient plus 6 control tissue. All TMAs were quality controlled by preparing H&E sections on the Symphony platform and inspection of histopathology by a pathologist.

**WP 5.2.** As a first marker LSD1 was analysed by means of IHC in the Targos lab on n=148 FFPE blocks of biopsies from colorectal cancer patients prior to neoadjuvant CRT and n=42 FFPE blocks from colorectal resection specimens post-treatment derived from the CAO/ARO/AIO-04 trial. As controls, n=40 resection specimens of patients not undergoing neo-adjuvant treatment have been stained by Targos technicians on the automated platforms Benchmark XT and Symphony

The results have been evaluated by a Targos pathologist and were evaluated statistically by a bio-statistician at Targos. No significant correlation to treatment response or any other clinical parameter could be established. The same was true for the analysis of PRK1.

In addition several antisera from WP1 were tested for use in IHC. None of the tested antibodies gave rise to specific staining results on FFPE tissue samples. The antibodies however were suitable for other techniques such as IP, ChIP or Western Blot, which were used in WP2.

Complementary to the antibodies from WP1 commercially available antibodies against candidate genes such as MLH1, MLH2, pHH3, PTEN or pERK were tested on the tumor samples. However no correlation with treatment results in the prospective CRC trial AIO-94 could be established.

Since in CRC cell culture panel (WP3) no significant correlation of any of the tested genes from WP1 with a specific genetic CRC subgroup could be established, no further efforts were undertaken to generate antibodies suited for use in FFPE.

**WP 5.3.** A spreadsheet data base for all available reagents within the consortium was generated to allow all consortium members to scan for interesting reagents relevant to their respective work. Further technical and clinical validation of any marker assays for diagnostic purpose was not performed for lack of scientific justification.

**Potential Impact:**

Tumour markers tests have been recently developed based on genomic technologies which have been shown by researchers and clinicians to have an important role in tumour progression. The work of several laboratories from USA and from Europe, including members of the EPIDIACAN consortium has elucidated mechanisms of epigenetic gene regulation, which can be exploited by the tumour biomarker field. Indeed, participant Laboratories of this network have analysed epigenetic markers in colorectal tumour (models) (Participants 1, 2) as well as in prostate and breast cancer (Participants 3, 6, 7). Other participant Labs have been contributing in basic knowledge and mechanistic analysis of epigenetic pathways and factors (Participants 4, 5). This consortium has assessed epigenetic factors and mechanisms, like histone modifications as well as modifying enzymes involved in these processes. Cancer models have been selected for the most part of the studies and results have been validated in clinical specimens. EPIDIACAN consortium contributes in identification of novel oncogene and tumour stage related epigenetic events and factors, in order to maximize the subsequent clinical exploitation as epigenetic markers. Candidate epigenetic markers as well as marker combinations have been tested in a panel of preclinical models (cell lines and transgenic mice). A tight management plan was adopted in order that the most efficient combination per tumour cell to reach the final evaluation in clinical samples. There is the challenge that all this basic mechanistic analysis of genetic vs. epigenetic pathways will be utilized to improve clinical practice and finally public health.

**Contribution to wider societal objectives**

The benefits on the society of a network focusing on novel early tumour markers are evident, since cancer is a major cause of death in Europe. This network contained groups of scientists from different disciplines like Molecular Biology, Epigenetics, Cell Biology, Tumour Biology, Biochemistry and Clinical Studies.

Bilateral interactions enhanced already successful co-operation by adding a value at the European level. Dissemination of the technologies developed at the individual national level will provide patients all over Europe to have access to new developments concerning cancer treatment, thus enhancing the benefit for the "European Citizen".

**Contribution to policy objectives of Health**

Cancer is the second major cause of mortality in Europe. A new era in human biology has been opened by the sequencing of the human genome offering opportunities to improve human health and to stimulate industrial activity. Our project represents a basic and applied research programme aimed to exploit the potential of genomic approaches to decipher mechanisms underlying genetic vs epigenetic mechanisms in oncogenesis. The goals of this proposed project were in accordance with the relevant objectives of the Health priority laid down in the 7th EU framework programme, because our research is focused on major fundamental biological processes concerning cell proliferation which can be exploited for novel tumour markers. Part of the work will therefore have direct implications on colorectal, prostate and breast cancer in humans through novel epigenetic markers for cancer diagnosis and cancer therapeutics. Further characterization of our already existing cell and mouse models for cancer on different genetic backgrounds allowed us to use these mice in preclinical trials and speed up the evaluation of novel markers in the clinic. New knowledge about tumour formation has eventually been translated to an application stage, to the design of new diagnostic tests

to detect cancer at an early stage and thus improve medicine in Europe and life quality.

It is the current understanding, that the majority of drug treatments is ineffective because the decision for a drug prescription is mainly driven by statistical results for a patient population, rather than a diagnostic result for an individual patient. In many cases the decision is not evidence based at all. This situation is becoming increasingly unacceptable, and hence addressing the challenge of predicting the response to a specific drug treatment is an imperative for three main reasons: First, selecting those patients who will have benefit of a drug from those who predictably will not benefit is highly ethical. Second, healthcare professionals and healthcare providers worldwide need diagnostic guidance on how to handle the mismatch between innovative high-value medicines and limited financial resources. Third, it is of economic advantage for the pharmaceutical industry to stratify patient populations in clinical drug development and stratification thus helps to increase medical innovation. For these three reasons, in March 2005, the U.S. Food and Drug Administration (FDA) issued a guidance that requests the submission of all available validated biomarker data for any application for a new drug. The European Medicines Agency (EMA) has its own regulations, which are sometimes even stricter than those of the FDA, as seen with the recent approval of the anti-cancer drug panitumab (Vectibix, Amgen Inc.), for which a diagnostic test ruling out therapy resistance is required in Europe but not in the USA.

Predictive Biomarkers Optimize Prescription. The instruments to address these challenges are validated predictive biomarkers. Unfortunately only few of these biomarkers have made their way to clinical practice, e.g. Her2 testing by immunohistochemistry or by in situ hybridization for the selection of breast cancer patients eligible for trastuzumab (Herceptin, Roche) treatment; HLA B5701 genotyping for the identification of HIV-infected patients most at risk for abacavir-related hypersensitivity reactions (Ziagen, GSK), the immunohistochemistry testing for the presence of estrogen receptors as prerequisite for antihormonal therapy in breast cancer patients or the analysis of K-RAS mutations by RT-PCR to identify therapy resistance to panitumumab in colon cancer patients. To improve this situation, many drug development companies have dramatically increased their efforts to co-develop predictive biomarkers together with new targeted therapies. Some pharmaceutical companies have joined forces with diagnostic companies, such as Eli Lilly Co. and GE Healthcare, in some cases the pharmaceutical and diagnostic branches of a company have started joint activities (e.g., Roche Pharma and Roche Diagnostics) with a clear focus on predictive pathology. The pharmaceutical industry's fear of fragmented markets due to patient stratification is diminishing, with more success stories such as Imatinib (Gleevec, Novartis) for small selected patient collectives.

### **Reinforcing European competitiveness**

EPIDIACAN has aspects in very important and competitive areas on preclinical models of cancer, cancer genetics and genomics, as well in imaging technologies. While a number of leading scientists within the field are active in Europe today, judging from the published literature it is evident that European research output lags behind that of the United States in production of efficient novel cancer diagnostics. The domination of US laboratories of the field has as a consequence the continuous drain of talented young European researchers towards the US. This consortium has established world leadership in the field, mainly due

to the competitive edge provided by EU support for intensive collaboration of a critical mass of researchers from different disciplines. The integrated approach has neutralized the problem of fragmentation of relevant expertise in Europe, thus translating the potential success of the project into tangible benefits for European science.

### **Transnational cooperation**

The complexity and the quantity of the proposed work demanded the collaboration of many laboratories possessing different expertise. Some of the participants (1, 4, 5) and (6, 7) had already initiated bilateral collaborations studying related topics. Although they had built up fruitful collaborations, these activities were fragmented and addressed specific aspects only. This project combined and expanded these individual activities to a more comprehensive collaboration. There were no chances to carry out such an ambitious project at the level of individual laboratories, or to gather the diverse but complementary expertise needed at the national level. Besides the combination of different expertise, the multidisciplinary nature of the program required the integration of resources available at the individual laboratories.

### **Impact on participating companies**

The project extended the service business model and thus increases the competitiveness of the participating Targos Molecular Pathology GmbH. The company had the opportunity to further explore own biomarker projects and build up own intellectual property within this project and to profit from the transfer of know-how from the collaboration with outstanding experts from different fields of life sciences and technology. Targos further improved its knowledge in the field of epigenetics on a tumour and patient specific manner and was able to use the network resources in order to improve its competitiveness in the market.

### **Unique training opportunities**

The participating laboratories had continuously open calls for positions for young European researchers. Although, training was not the prime objective of this application, given the cutting-edge scientific level of research to be performed and the opportunity to carry-out research projects in collaboration with laboratories of a different discipline, the network provided a competitive alternative to US laboratories for training young researchers interested in the field of cancer epigenetics. Via short-term visits, the young fellows had an opportunity to work in more than one laboratory. The opportunity to meet and collaborate with several network members and to be exposed to the continuous information exchange of the whole network has set up a precedent of a novel, highly productive training method highly attractive to young researchers interested in the field.

### **WIMM**

The main dissemination for us was publication and presentations at various scientific meetings and seminars. The potential impact is to improved diagnosis and treatment of colorectal cancer. At the stage we have no immediate plans for exploitation of results, though there may be an opportunity for doing something with the development of effective cultures from fresh tumour material. We have also obtained additional information on the colorectal cancer cell line panel which is useful for assessing in vitro drug response assay results in relation to the properties of a cancer.

Our finding that lumen formation in vitro and in vivo is representative of cancer stem cell differentiation will refine the classical pathologist interpretation of colorectal tumours. Pathologist grade tumours according to the glandular formation and this forms part of the prognosis. Our study shows that tumours and cell lines can be classified into two broad groups: lumen forming and non-lumen forming. The formation of lumens clearly demonstrated that a particular line or tumour is differentiating along the enterocyte lineage. Differentiation decreases the number of dividing cells, and as such lumen forming tumours grow and metastasise slower. Tumours that fail to form lumens are not undergoing significant differentiation and therefore most cells remain stem like and replicate at a higher rate, hence have a worse prognosis. Our results indicate that the transcription factor CDX1, which is frequently silenced in tumours, may be important for the formation of lumens and hence tumour differentiation.

We have also developed a efficient method to grow primary tumours from biopsies and endoscopies and this has the potential to significantly improve diagnosis and tumour classification. The health implications of this approach are potentially large as anti-cancer treatments can now be applied to growing samples derived from a much wide variety of patients. There is scope that our approach may allow some personalised treatments to be tested in vitro for efficacy, for example, antibody mediated immune killing using the patient's own blood.

Furthermore in vitro studies of tumour differentiation may also be used to refine the classical pathological definition of differentiation. The potential cost benefit for EU healthcare may be substantial as drug treatments can be refined and suitable drug regimes tailored to suit each individual patient.

## **IPOFG**

The findings are expected to originate at least six articles in the course of the project, which will be submitted for publication in international, peer-reviewed journals. More publications are expected when the long-term objectives are accomplished. Furthermore, the findings will be presented in local seminars, and in several national and international conferences in which the team investigators participate, including: Workshop on Molecular Genetics of Human Solid Tumors, Meetings of the European Association for Cancer Research and of the Portuguese Society of Human Genetics, Congress of the Portuguese Oncology Society, European Congress of Pathology and the Workshop on Medical Oncology organized by our Institution.

Three members of the research team lecture in graduate and postgraduate courses mainly for medical students and medical doctors at the University of Porto, enabling transmission of knowledge and stimulation of future researchers. Significant findings will be publicized through the media, in order to contribute to science education and awareness in society.

Societal implications. The prostate is the most common site of malignant transformation in western men. About 30% of men older than 50 years in the western countries develop prostate adenocarcinoma and the lifetime risk of clinical disease is 10%, with the risk of mortality being around 3%. The great variation in clinical behavior of prostate cancer creates a major dilemma in the treatment-decision process, since not all men with

microscopic carcinoma require aggressive radical therapy. The major difficulty is the present-day absence of reliable tools to predict which cancers will remain indolent and which are going to kill the patient if left untreated. This project identified epigenetic markers of biological aggressiveness in prostate cancer and this information may have a wide impact on health and socioeconomics, both by reducing over-treatment and by allowing concentration of resources to treat intensively those patients who actually need it.

**List of Websites:**

<http://www.eie.gr/nhrf/institutes/ibrb/eu-projects/epidiacan/index-en.html>