The Belgian Society for Cell and Developmental Biology

Spring Meeting 2007

"Chromatin Dynamics and Epigenetics"

Liège, March 10, 2007

Invited speakers

G. ALMOUZNI
(Paris, France)

I. DAVIDSON
(Strasbourg, France)

F. FUKS
(Brussels, Belgium)

R. METIVIER
(Rennes, France)

R. SHIEKHATTAR
(Barcelona, Spain)

Information on meeting

http://bscdb.UGent.be

Deadline for abstract submission

February 15, 2007

Contact

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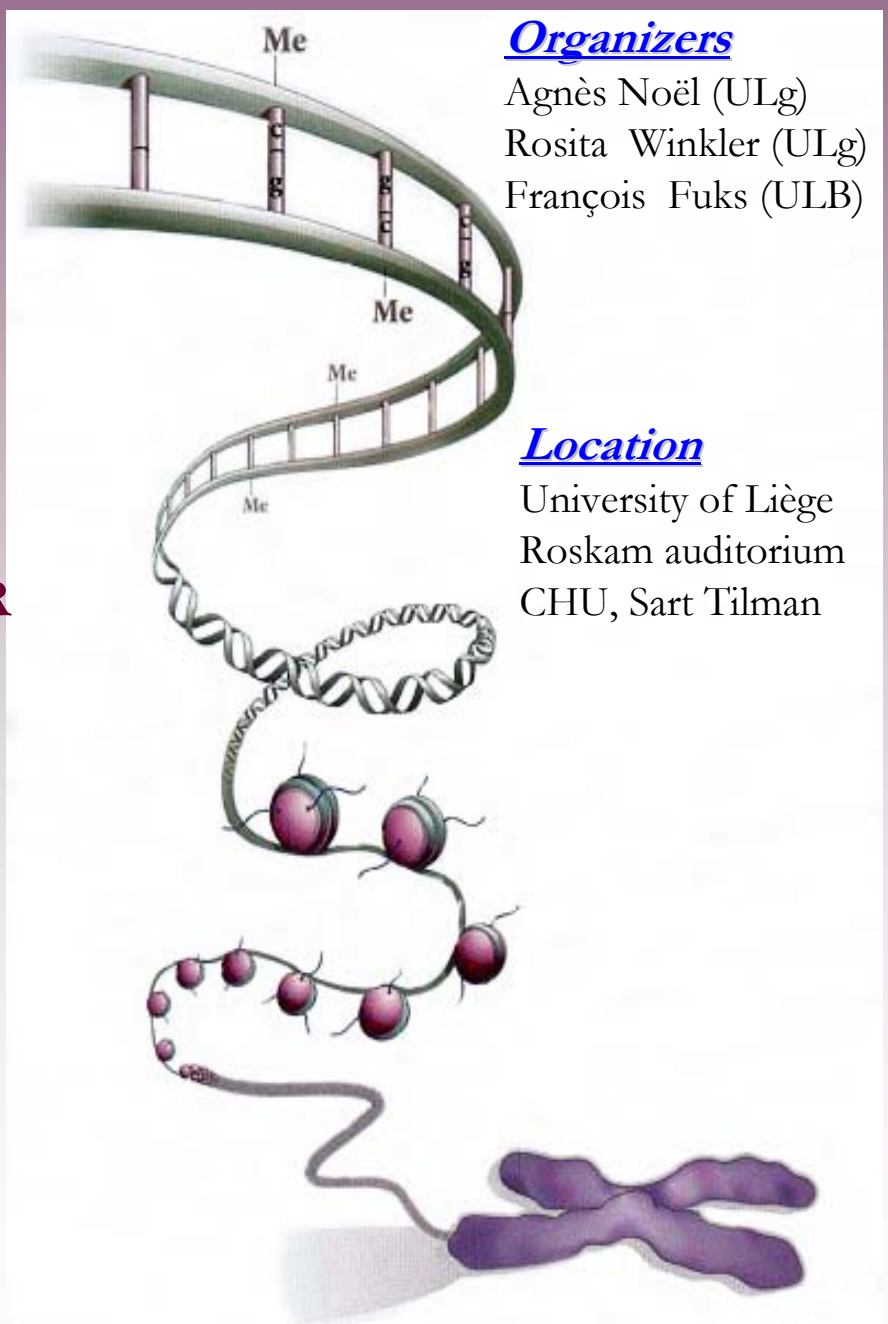

From « A Review of the Universe - Structures, Evolutions, Observations, and Theories »
http://universe-review.ca

Organizers

Agnès Noël (ULg)
Rosita Winkler (ULg)
François Fuks (ULB)

Location

University of Liège
Roskam auditorium
CHU, Sart Tilman

POSTER PRIZES

At the end of the Spring Meeting 2007, two poster prizes were awarded to:

HISTON DEACETYLASES IN HEPATIC STELLATE CELLS: A PRELIMINARY STUDY

Nele R. Nuytten, Inge Mannaerts, Albert Geerts and Leo A. van Grunsven

Laboratory for Cell Biology (CYTO), Faculty of Medicine and Pharmacy, Free University of Brussels (VUB), B-1090 Brussels

and

THE TRANSCRIPTIONAL REPRESSOR NIPP1 IS A NOVEL PLAYER IN EZH2-MEDIATED GENE SILENCING

Mieke Nuytten, Lijs Beke, Aleyde Van Eynde, Monique Beullens and Mathieu Bollen

Laboratory of Biosignalling & Therapeutics, Departement of Molecular Cell biology, Faculty of Medicine, KULeuven, B-3000 Leuven

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Spring Meeting of the Belgian Society for Cell and Developmental Biology

"Chromatin dynamics and epigenetics"

Liège, March 10, 2007
C.H.U., Roskam auditorium, Sart Tilman

Program

8H30 - 9H15	Registration and display of posters
9H15 - 9H30	Welcome and introduction
MORNING SESSION	
9H30 – 10H15	**Speaker 1: F. Fuks** (Brussels, Belgium): "Mechanisms of DNA methylation in mammals"
10H15 – 11H00	**Speaker 2: R. Metivier** (Rennes, France): "Dynamic of ER-mediated transcriptional activation of responsive genes in vivo"
11H00 – 11H30	Coffee break and visit of commercial exhibitions
11H30 – 12H15	**Speaker 3: I. Davidson** (Strasbourg, France): "Role of TFIID subunits TAF4 and TBP in signaling pathways and cell proliferation"
12H15 – 12H45	Two presentations by young investigators
12H45 – 14H00	Lunch – Poster viewing – Visit of commercial exhibitions
AFTERNOON SESSION	
14H00 – 14H45	Two presentations by young investigators
14H45 – 15H30	**Speaker 4: G. Almouzni** (Paris, France): "Chromatin assembly factors, histone H3 variants and cell cycle"
15H30 – 16H15	**Speaker 5: R. Shiekhattar** (Barcelona, Spain) : "Silencing of the genome through histone demethylation"
16H15 – 16H30	Poster Awards
END OF THE MEETING	

Organizers : Prof. R. Winkler (ULg) – Prof. A. Noël (ULg) – Dr F. Fuks (ULB)

Information, Registration and Abstract Submission : http://bscdb.UGent.be

Fuks François : presenting author – e-mail :

MECHANISMS OF DNA METHYLATION IN MAMMALS

François Fuks

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In mammals, DNA methylation plays an important role in development and is associated with transcriptional silencing. The goal of our current work is to elucidate the mechanisms by which the DNA methylation machinery - the DNA methyltransferases (DNMTs) and the MBDs- functions. In particular, we wish to address two questions: 1. How are the DNMTs targeted to specific genomic sequences? 2. How do the DNMTs and the MBDs lead to gene silencing?

1. Our recent work suggests that the DNMTs can be recruited to particular loci through their association with specific transcription factors. Our studies suggest that targeting of DNA methyltransferases by transcription factors could be a general mechanism by which specific DNA methylation patterns are generated.

2. One mechanism by which the DNA methylation machinery brings about transcriptional repression is through recruitment of HDAC and histone H3 Lys9 methyltransferase activities. Our recent work indicates that the DNA methylation machinery is mechanistically linked to the Polycomb Group (PcG) proteins. On the one hand, our results suggest that EZH2 serves as a recruitment platform for DNA methyltransferases. On the other hand, our more recent work indicates that conversely, CpG methylation can influence EZH2 function through the methyl-CpG-binding protein MeCP2. Thus, the link between EZH2 and DNA methylation is a two-way connection. Our results provide evidence that MeCP2 acts as a bridge between two essential epigenetic systems, DNA methylation and PcG proteins. Moreover, this study reveals a novel mechanism for EZH2 recruitment to mammalian promoters, a process about which very little is known.

References

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2. Brenner et al. EMBO J. 2005 24:336-46.

3. Vire et al. Nature 2006 439:871-4.

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ROLES OF TFIID SUBUNITS TAF4 AND TBP IN CONTROL OF CELL PROLIFERATION

Anas Fadloun, Dominique Kobi, Laurence Delacroix, Aurore Morlon, Mohamed-Amin Choukrallah, Isabelle Michel, Gabrielle Mengus and Irwin Davidson

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General transcription factor TFIID comprises the TATA-binding protein TBP and 13 TBP-associated factors, TAFs. Present at most if not all Pol II transcribed promoters, TFIID may be a platform to integrate different signals and modulate gene expression. We have addressed the roles of TBP and TAF4 in signalling pathways and control of cell proliferation. Inactivation of murine TAF4 in embryonic fibroblasts results in constitutive activation of the TGFbeta-signalling pathway promoting serum-independent autocrine growth. Our results also show that TAF4 is a critical cofactor for the retinoic acid (T-RA) receptor (RAR), being required for T-RA regulation of most, but not all cellular genes in fibroblasts. We have further found that T-RA induces TGFbeta-dependent autocrine growth by directly inducing the secondary cytokine CTGF (connective tissue growth factor) via a conserved DR2 RARE in its proximal promoter. By comparing transcriptomes, we identify a set of genes that are induced or repressed by loss of TAF4 and by T-RA corresponding to the genetic programme of a novel cross-talk between the T-RA and TGFbeta pathways that leads to deregulated cell growth.

We have also developed tbplox/- fibroblasts where TBP can be inactivated by expression of the Cre recombinase leading to cell death. We have used these cells to test the ability of 19 different mutants of TBP to complement loss of the endogenous protein. Many of the mutated TBPs retain the ability to rescue cell viability, but lead to impaired growth. We have characterised in detail one of the cell lines expressing a mutated TBP. We show that the mutated TBP fails to interact with one of is partners in vivo leading to up or down-regulation of a specific set of genes. ChIP assays show that the mutation does not affect TBP promoter binding, but rather differentially affects subsequent steps in preinitiation complex formation on these target genes. (project in collaboration with Pim Pijnappel and Marc Timmers, University Medical Center, Utrecht Holland).

Almouzni Geneviève : presenting author – e-mail :

CHROMATIN ASSEMBLY FACTORS, HISTONE H3 VARIANTS AND CELL CYCLE

Dominique Ray-Gallet, Sophie Polo, Jean-Pierre Quivy, Anja Groth, Danièle Roche and Geneviève Almouzni

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The ordered assembly of chromatin produces a nucleoprotein template capable of regulating the expression and maintenance of the genome functions.

Factors have been isolated from cell extracts that stimulate early steps in chromatin assembly in vitro. One such factor, chromatin assembly factor-1 (CAF-1), facilitates nucleosome formation coupled to DNA synthesis. It is thought to participate in a marking system at the crossroads of DNA replication and repair to monitor genome integrity and to define particular epigenetic states. We have begun to approach its critical importance during early development in Xenopus laevis and using mammalian cell systems. In addition, we have now identified a chromatin assembly pathway independent of DNA synthesis. The HIRA protein appears critical for this pathway in Xenopus egg extracts. Notably, CAF-1 was part of the the histone H3 complex, H3.1 complex (replicative form) and HIRA of the H3.3 complex (replacement form) (Tagami et al, 2004, Nakatani et al, 2004). A major goal in our laboratory is now to better integrate the function of these factors in vivo during development and also in connection with replication, repair and control of histone pools.

We will discuss our recent findings on this topics and the interrelationships with other assembly factors.

References

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Polo S. & Almouzni G. (2006) Chromatin assembly : a basic recipe with various flavors. Current Opinion in Genetics and Development, 16, 104-111.

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Shiekhattar Ramin : presenting author – e-mail :

GENE EXPRESSION AND REGULATION PROGRAM AND MOLECULAR AND CELLULAR ONCOGENESIS PROGRAM

Ramin Shiekhattar

CRG, Barcelona, Spain

Histone methylation is a post-transcriptional mark regulating chromatin structure and gene regulation. Once deemed irreversible, recent findings have identified two classes of enzymes capable of demethylating lysine residues. BHC110/LSD1, the catalytic heart of multiple co-repressor complexes, was the first of such demethylases shown to reverse dimethyl histone H3 lysine 4 (H3K4). However, due to intrinsic limitations of BHC110/LSD1 mode of action, it is unable to remove trimethyl H3K4 marks. Here we show that JARID1d, a JmjC domain-containing protein, specifically demethylates trimethyl H3K4.

Detailed mapping analysis revealed that besides the JmjC-domain, the BRIGHT and zinc-finger-like C5HC2 domains are required for maximum catalytic activity. Importantly, isolation of native JARID1d complexes from human cells revealed the association of the demethylase with a polycomb-like protein Ring6a/MBLR. Ring6a/MBLR not only directly interacts with JARID1d but also regulates its enzymatic activity. We show that JARID1d and Ring6a occupy human Engrailed 2 promoter and regulates its expression and H3K4 methylation levels. Depletion of JARID1d not only results in increased trimethylated H3K4 but also enhanced recruitment of chromatin remodeling complex, NURF, and the basal transcription machinery near the transcriptional start site revealing a role for JARID1d in regulation of transcriptional initiation through H3K4 demethylation.

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HIGH FREQUENCY OF RASSF1A AND RARB2 GENE PROMOTER METHYLATION IN MORPHOLOGICALLY NORMAL ENDOMETRIUM ADJACENT TO ENDOMETRIOID ADENOCARCINOMA

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Background: Endometrioid carcinoma of the endometrium (EEC) accounts for about 80% of endometrial carcinoma cases and results from of the accumulation of several genetic abnormalities and hormonal imbalances. The purpose of this study was to search for a DNA methylation signature of EEC in the early steps of endometrial carcinogenesis.

Methods: Archival biopsies of 39 EECs, 14 atypical hyperplasia (AH), 11 histologically normal endometrial tissues adjacent to EECs and 24 normal control endometrium of non cancerous patients were retrieved. After DNA extraction, the cases were tested by quantitative methylation specific PCR (QMSP) with primers hybridizing in the promoter regions of five genes frequently methylated in human cancer (RASSF1A, RARb2, P16, MGMT and GSTPi). Results: 29/39 (74%) of the EECs and 7/14 (50%) of the AHs were methylated for RASSF1A gene whereas 17/39 (44%) of the EECs and 6/14 (43%) of the AHs were positive for the methylation of RARb2 gene. No significant results were obtained for the other genes (P16, MGMT and GSTPi). Interestingly, 4/11 (36%) and 6/11 (55%) of normal endometrial tissues adjacent to EEC showed, respectively, RASSF1A and RARb2 gene methylation. There was no significant rise in the mean labelling indices for Ki 67, cyclin D1 and apoptosis for these 11 cases compared to the controls. In addition, these tissues were found to be microsatellite stable (MSS) in contrast to EECs where an instability was detected in 2/5 (40%) of cases.

Conclusion: Promoter region methylation of RASSF1A and RARb2 genes is an early event during the endometrial carcinogenesis.

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CANINE MAST CELL TUMOURS: ULTRASTRUCTURAL STUDY AND THE ROLE OF MCM-7 AS A PROGNOSTIC MARKER

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Introduction – Mast cell tumours (MCTs), which are one of the most common neoplasms in dogs, show a very diverse clinical behaviour and hence a difficult predictable prognosis. Until now, prognosis is mainly evaluated by application of the Patnaik histological grading system, based on the differentiation grade of the tumour. However this histopathological system has its limitations. In the last years many studies have been performed in an attempt to predict the biological behaviour of MCTs in dogs. The most frequently used prognostic marker is a set of proliferation markers Ki67/PCNA/AgNOR. Minichromosome Maintenance (MCM) markers are a new set of proliferation markers, whose prognostic value has already been demonstrated in different human tumours. In animals its application has until now been restricted to rats, mice and flies. In the past several studies already described the ultrastructural features of the different MCT grades. The prognostic significance of ultrastructural characteristics of these tumours is however less well known.

Aim – The aim of this study is two-fold:

1. an immunohistochemical (IHC) analysis of Ki67 and MCM7 on MCTs

2. a review of the most important ultrastructural findings of MCTs

Materials and methods – Samples, which consisted of formalin-fixed paraffin-embedded material from 4 canine MCTs, were sectioned at 6µm thickness and immunohistochemically stained for MCM7 and Ki67. For the ultrastructural study, sections 40–60nm thick are cut, collected on 50 mesh copper grids and examined using transmission electron microscopy (TEM).

Results – The IHC analysis reveals no or faint staining of the proliferation marker Ki67 in the tumour. Although the staining of MCM7, present in each tumour biopsy, is variable, the intensity of positivity is stronger compared to Ki67.

The ultrastructural study shows not only the usual array of mast cell organelles, but reveals also less usual findings, of which the most important are: complex interdigitations between cytoplasmic projections of adjacent mast cells, dense parallel stacks of 10nm-filaments (sometimes in combination with parallel arrays of tubules) and the presence of invaginated mast cell nuclei. Furthermore we observed a very diverse morphology of secretory granules and the presence of blood vessels, fibrillar collagen and other inflammatory cells in the tumour stroma.

Conclusions – The IHC results might indicate that MCM-markers are more sensitive markers of proliferation compared to Ki67. However future research is necessary to confirm this on a larger series, in which their value as prognostic marker in canine MCTs may also be evaluated. TEM analysis confirms the results of the study of Madewell et al. By determining the differentiation grade of a tumour based on its ultrastructural features, it may be possible in the future to use this method as a prognostic indicator.

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X-LINKED MENTAL RETARDATION AND EPIGENETICS

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Mental retardation (MR) is a common (2-3%) non-progressive cognitive impairment mostly affecting normal brain development. X-linked gene defects have been considered as an important cause of MR, because more males than females are suffering from MR (XLMR).

Combined efforts in molecular genetics, positional cloning and mutation screening already led to the identification of tens of MR-related genes on the X chromosome. More recently, microarray-Comparative Genomic Hybridization (array-CGH) allowed for the detection of disease-associated copy number alterations of dosage-sensitive genes, adding gene duplication as a novel mechanism to explain the MR phenotype. For a significant percentage of XLMR patients however, the underlying cause has not been resolved, strongly suggesting that yet unidentified mechanisms could play important roles in their aetiology. We hypothesise that modifications of the epigenetic profile at specific loci in the genome might represent such a mechanism.

Chromatin immunoprecipitation (ChIP) is a powerful method to investigate epigenetic marks at a locus-specific (by qPCR) or whole genome level (by ChIP-on-chip). In order to introduce this technique in our lab we first analysed such marks at the FMR1 locus of fragile-X patients. We could clearly detect increased promoter DNA methylation as well as the expected histone modifications (MeH3K4, AcH4 and AcH3) in cells derived from these patients. In addition, increased levels of DNA methylation were found for several loci on the X chromosome in females compared to males. In a second step, we anticipate to perform X-chromosome-directed epigenetic profiling of XLMR patients using X chromosome-specific high-resolution oligo arrays that cover all X genes as well as their regulatory sequences. This might allow us to define epigenetic modifications as a novel mechanism that disturbs normal cognitive development, or might result in the identification of novel XLMR genes.

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COMBINED IMMUNODETECTION OF AP-2 AND YY1 TRANSCRIPTION FACTORS IS ASSOCIATED WITH HER2 GENE OVEREXPRESSION IN PRIMARY BREAST TUMORS

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§: contributed equally

Overexpression of the HER2 oncogene is observed in about 30 percent of human breast cancers and is generally correlated with a poor prognosis. This overexresssion is the consequence of increased transcription rates, frequently associated with gene amplification.

Several in vitro and in vivo data showed a link between AP-2 transcription factors and the transcription of the HER2 oncogene in cancer cell lines. Moreover, the Yin Yang 1 (YY1) co-factor has been shown to stimulate AP-2 transcriptional activity on the HER2 promoter in cancer cell lines.

In this report, we examined the relationship between AP-2alpha, YY1 and HER2 both in breast tumors and cell lines.

Using immunohistochemistry, we observed a statistically significant correlation between HER2 and AP-2alpha expression. We also found an association between HER2 expression and the combined high expression of AP-2 and YY1 partners. Moreover, a strong correlation between AP-2alpha and YY1 transcription factors expressions was also observed.

In a breast cancer cell line, upon transfection of siRNAs designed against AP-2 mRNAs, we detected a decrease in endogenous HER2 expression. Moreover, addition of siRNA directed against YY1 further reduced the HER2 protein level, suggesting the important cooperation between AP-2 and YY1 transcription factors for HER2 expression.

In addition, an association between both AP-2 and YY1, and the estrogen (ER) and progesterone (PR) receptors expressions was found in primary breast tumors. Moreover, estrogen treatment induced an increase in AP-2alpha, -gamma and YY1 endogenous expressions in breast cancer cell lines.

In conclusion, our data highlight the roles of both AP-2 and YY1 transcription factors in HER2 oncogene overexpression in breast cancers. It is also the first time that a link between YY1 and hormonal receptors expressions is detected in primary breast tumors.

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ANGIOGENIC ACTIVITY OF HUMAN CHORIONIC GONADOTROPIN (hCG) THROUGH LH RECEPTOR ACTIVATION ON ENDOTHELIAL AND EPITHELIAL CELLS OF THE ENDOMETRIUM

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Successful embryo development requires an extensive endometrial angiogenesis in proximity of implantation site. HCG is a glycoprotein that is produced by trophoblast tissue very early in normal pregnancy and also in trophoblastic disease. In trophoblastic tumors (choriocarcinomas), hCG is produced in large amounts and seem to play a key role in their angiogenic and metastatic phenotype.

In this work, we demonstrate an angiogenic effect of hCG in several in vivo (Chick chorio-allantoïc membrane, matrigel plug assay, aortic ring assay) and in vitro experimental models. In contrast human placental lactogen (hPL) did not display angiogenic properties.

LH/hCG receptor was detected in endothelial cells by RT-PCR and by Western blotting. In mice aortic ring assay, angiostimulation by hCG was abrogated by deletion of LH/hCG receptor (LuRKO mice). Use of recombinant hCG and anti-hCG antibody further confirmed the specificity of this angiogenic activity. By using dibutyryl cAMP, adenylate cyclase or protein kinase A inhibitors, we demonstrate that hCG-mediated angiogenesis involves adenylyl-cyclase – protein kinase A activation. Addition of hCG to endometrial epithelial epithelial cells, but not to cultured endothelial cells stimulated vascular endothelial growth factor (VEGF). VEGF and hCG also displayed additive activities. Altogether, these data demonstrate that peritrophoblastic angiostimulation may result from a paracrine dialogue between trophoblast, epithelial and endothelial cells through hCG and VEGF.

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THE ARABIDOPSIS HOMOLOG OF YEAST BRE1 HAS FUNCTION IN CELL CYCLE REGULATION DURING EARLY ORGAN GROWTH

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Histone modification and transcriptional activation are unconventional roles for ubiquitin E3 ligases that are usually associated with protein degradation. We have identified an Arabidopsis HCa-RING type ubiquitin E3 ligase as HISTONE MONOUBIQUITINATION protein1 (AtHUB1, and its homolog AtHUB2). AtHUB1 is an homolog of human and yeast BRE1 proteins. AtHUB1 monoubiquitinated histone H2B in vitro and genetic interaction studies confirmed that AtHUB1 and AtHUB2 act in the same pathway. Hub knockout mutants had pale leaf coloration, modified leaf shape, reduced rosette biomass, and inhibited primary root growth, suggesting that they have a role in organ growth. Kinematic analysis of leaf and root growth showed that hub1-1 mutation increased cell cycle duration in young leaves and resulted in reduced cell numbers and size. Flow cytometric analysis revealed a cell cycle block at G2-to-M transition and increased endoreduplication levels. Furthermore, transcript profiling of shoot apical meristems of hub1-1 showed that key regulators of the G2 to M transition were misexpressed in the mutant. Based on the mutant characterization, we propose a function for HUB1 in cell cycle control through histone modifications that may be involved in transcriptional regulation.

The Arabidopsis ANGUSTA4 is an homolog of yeast BRE1 and has a function in cell cycle regulation during early leaf and root growth.

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THE LYMPHATIC RING ASSAY: A 3D CULTURE SYSTEM FOR DISCOVERING NOVEL GENES AND MECHANISMS THAT REGULATE LYMPHANGIOGENESIS

Françoise Bruyère, Laurence Melen-Lamalle, Silvia Blacher, Guy Roland, Marc Thiry, Jean-Michel Foidart and Agnès Noël

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Blood vessels and lymphatic vessels form a system for circulation of fluids and delivery of molecules within the body. Despite its implication in numerous pathologies, the lymphatic system has until recently been overshadowed by the greater emphasis placed on the blood vascular system. Study on lympangiogenesis, the recruitment of new lymphatic vessels in tumor is hampered by the lack of reproducible ex vivo or in vitro model. In order to overcome the lack of specific lymphatic vessel culture system to study lymphatic vessels, we have developed a new and original in vitro model. We successfully transposed the three-dimensional aorta ring assay to a mouse lymphatic thoracic duct assay. Fragments of thoracic duct isolated from mice are embedded into a collagen gel and cultured for two weeks. By immunochemistry and transmission electron microscopy, we characterized the outgrowing cells as being lymphatic cells that organize into microvessels containing a lumen. A computer-assisted quantification system has been developed to quantify lymphatic cell outgrowth. Our studies using this model confirmed that VEGF-C and PDGF-BB stimulated lymphangiogenesis, but VEGF-A, FGF2 and PlGF did not. Though plasminogen activator inhibitor-1 (PAI-1) was critical for angiogenesis, it was dispensable for lymphatic outgrowth.

The lymphatic ring assay offers new opportunities for rapid identification of unknown regulators of lymphangiogenesis by testing activators and inhibitors as well as by using transgenic KO mice.

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VASCULAR ARCHITECTURE OF BREAST CANCER XENOGRAFTS OVER-EXPRESSING MT4-MMP

Vincent Chabottaux (1), Marc Thiry (2), Lorin Host (1) and Agnès Noël (1)

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2: Laboratory of Cell and Tissue Biology, University of Liège, B-4000 Liège

Our previous data show that MT4-MMP, a membrane-anchored MMP essentially expressed by breast tumor cells, increases breast cancer growth with frequent enlargements of intra-tumor blood vessels and promotes lung metastases (§).

In order to understand the way by which MT4-MMP affects the tumor progression, we have performed ultrastructural, immunohistological and gene expression profile studies of MDA-MB-231 breast cancer xenografts expressing or not MT4-MMP in mice.

Transmission electron microscopy and immunohistological analyses show that most of the intra-tumoral blood vessels are covered by pericytes in both conditions. However, in the presence of MT4-MMP, mural cells (alpha-SMA positive) are more frequently detached from the endothelium with irregular shapes and extend irregular cytoplasmic processes in contact or not with endothelial cells. Moreover, basement membranes of these vessels are more often degraded or absent in MT4-MMP condition.

Interestingly, super-array and RT-PCR studies reveal that human Thrombospondin-2 (TSP-2) expression is down-regulated in xenografts expressing MT4-MMP. The reduced expression of this anti-angiogenic factor has already been associated with disrupting of vascular integrity and permeability in mouse models. Moreover, its expression is associated with a diminution of the vessel size, with an inhibition of tumor growth in mice and with an inhibition of metastasis in human cancer.

This suggests that MT4-MMP could facilitate tumor growth and metastasis by disturbing the vascular integrity via the down-regulation of TSP-2. Further studies are however required to assessed the vascular leakage in xenografts and to determine whether MT4-MMP is directly or not implicated in the regulation of this anti-angiogenic factor.

§: Chabottaux et al. Cancer Res. 2006;66(10):5165-72.

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A ROLE FOR TANK/I-TRAF IN THE TLR-DEPENDENT SIGNALLING PATHWAYS

Jean-Stéphane Gatot (1,2,§), Romain Gioia (1,2,§), Tieu-Lan Chau (1,2,§), Michael Warnier (1,2), Jean-Paul Chapelle (1,2), Marie-Paule Merville (1,2), Keith Brown (5), Ulrich Siebenlist (5), Eric Muraille (4), Emmanuel Dejardin (1,3), Jacques Piette (1,3) and Alain Chariot (1,2)

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Innate immunity in response to a variety of viral and bacterial pathogens is established upon binding of their molecular components on specific receptors of the TLR family and subsequent activation of transcription factors such as IRF3/7 and NF-kappaB. These latter proteins, once activated through phosphorylation, play critical roles for the transcriptional induction of the type I interferon (IFN) genes, a critical event for innate immunity. The IKK-related kinases IKKepsilon and TBK1 have been identified as the IRF3/7 phosphorylating proteins but how these kinases are assembled into functional complexes remains unclear. TANK/I-TRAF has been described as an TBK1/IKKepsilon-interacting protein but its role in the TBK1-dependent signalling pathways has not been established yet. To address this issue, we performed yeast-two-hybrid analyses and now show that TANK/I-TRAF plays a role in the TLR-mediated IRF3/7 activation pathways. Moreover, we also show that TANK/I-TRAF is subjected to post-translational modifications, including polyubiquitination. What is the relevance of these modifications of TANK/I-TRAF in the context of the TLR-dependent-signalling pathways will be discussed.

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A ROLE FOR THE TRANSCRIPTIONAL COMPLEX ELONGATOR IN CELL MIGRATION AND SPREADING.

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Elongator is a multi-protein complex required for the transcriptional elongation of a subset of target genes. This six subunits complex (hELP1-6) is assembled by the scaffold protein, hELP1/IKAP and harbours an intrinsic histone acetyltransferase activity required for progression through the nucleosomes. Loss of Elongator/hELP1 function causes Familial Dysautonomia (FD), a human genetic disease characterized by neurodevelopmental defects and progressive degeneration of the sensory and autonomous nervous system. This loss of function is due to a point mutation within a splicing site of the IkBKAP gene which ultimately leads to the synthesis of an unstable transcript. As a result, cells from FD patients have decreased hELP1/IKAP expression. We recently demonstrated that cells depleted in hELP1/IKAP have defects in cell migration because a subset of genes coding for proteins required for cell motility are not properly transcribed. Still, why cell migration is altered upon hELP1/IKAP depletion is unclear. Here, we describe the generation of new cellular models where hELP1/IKAP is specifically depleted by RNA interference. We now demonstrate that Elongator is required for cell spreading and we began to elucidate the underlying mechanisms. Our results therefore highlight cell migration as a critical process whose disruption may underlie FD. Moreover, we also established a functional link between cell motility and transcriptional elongation.

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CYCLICAL RECRUITMENT OF THE HISTONE DEIMINASE PADI4 AND HDAC1 ON A NATURAL ESTROGEN TARGET PROMOTER

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Covalent histone modifications play an important role in regulating chromatin dynamics and function. One such modification, methylation, occurs on both lysine and arginine residues and participates in a diverse range of biological processes including heterochromatin formation, X-chromosome inactivation and transcriptional regulation. Until recently, it was unclear whether enzymes capable of antagonizing histone methylation existed. However, recent studies have revealed that histone methylation can be reversed by several histone demethylases, including PADI4, LSD1 and JmjC domain-containing demethylases. PADI4 (peptidylarginine deiminase 4) was the first to be identified; it functions as a histone deiminase that converts mono-methylated arginine to citrulline at specific sites of the tail of histones H3 and H4. This activity of PADI4 is linked to the repression of an oestrogen-responsive gene, pS2.

To investigate the mechanisms by which histone demethylation and in particular PADI4 functions, and as no PADI4 interactors have been described so far, in vitro interaction assays and coimmunoprecipitations were performed. We found that the histone deacetylase HDAC1 interacts with PADI4 and associates with PADI4-mediated histone deiminase activity.

Next, we investigated whether PADI4 and HDAC1 could be concomitantly recruited to the pS2 target gene promoter. It is already known that in presence of estrogen, the expression of pS2 is controlled by the Estrogen Receptor alpha (ER) that cycles on its promoter. Not only is the receptor cycling, but also follows a sequence of recruitment of co-factors which ultimately results in the binding of RNA polymerase II. We performed Chromatin immunoprecipitations (ChIPs) in MCF7 cells using antibodies against PADI4, HDAC1, citrulline H3 and acetylated histones. These ChIP assays revealed that both PADI4 and HDAC1, as well as their corresponding enzymatic activities, are cyclically associated with the pS2 promoter during the repression phases. Moreover, sequential ChIP (Re-ChIP) experiments showed that PADI4 and HDAC1 assembled together on the pS2 promoter.

Overall, our data allowed to better understand how histone deimination by PADI4 represses gene expression. In particular, our work suggests that gene silencing of the pS2 promoter requires the coordinated action of deimination and deacetylation of histones.

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COMPETITIVE BINDING OF RHOGTPASES TO RHOGDI REGULATES THEIR STABILITY AND ACTIVITY

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The small GTPases of the Rho family are at the crossroads of signaling pathways initiated by receptors to diffusible biological mediators or by cell-adhesion receptors. They are key signaling molecules regulating various aspects of the cellular phenotype such as proliferation, apoptosis, migration, cytoskeleton organization or gene expression. They shuttle between an inactive GDP-bound-state and an active GTP-bound state. Their level of activation is regulated by three classes of factors: the GEFs that catalyze the exchange of GDP to GTP, the GAPs which increase the intrinsic GTPase activity of the RhoGTPase and the RhoGDIs that bind the GDP-bound RhoGTPases and inhibit the exchange of GDP to GTP. We hypothesized that the complete depletion of a particular RhoGTPase isoform should reveal novel mechanisms of regulation in this complex network undetectable with the usual and less specific techniques. The Rho sub-class includes RhoA, its closely related homolog RhoC and RhoB a short-lived protein barely detectable in basal conditions. In human primary dermal fibroblasts and in various human cell lines the silencing of RhoA induced a strong increase of RhoB protein level. RhoB concentration was also slightly increased upon RhoC silencing while neither Rac1 nor Cdc42 silencing affected RhoB expression. Similar results were obtained by using two different siRNA sequences and the RhoB up-regulation was rescued by re-expressing a wild-type RhoA encoded by a construct bearing silent mutations impeding its recognition by the siRNA. Pull-down assay demonstrated that RhoB induced by RhoA and/or RhoC silencing was active while the simultaneous knockdown of RhoA, RhoC and RhoB revealed a role for RhoB in actin organization. The up-regulation of RhoB by silencing RhoA and/or RhoC was not related to an increase of its mRNA but to an increased half-life of the protein. Several studies suggest that RhoGTPases are protected from degradation by binding to RhoGDIs. Knocking-down RhoGDIalpha antagonized the increase of RhoB protein level induced by RhoA silencing while the forced over-expression of RhoGDIalpha alone was sufficient to increase RhoB. These results suggest that, in basal conditions, RhoGDIalpha is rate-limiting and the suppression of RhoA makes it available to stabilize RhoB. This competition for RhoGDI binding was not limited to members of the Rho sub-class. The double silencing of RhoA and RhoC with two different sets of siRNAs simultaneously increased the binding of Rac1 to RhoGDIalpha as observed by co-immunoprecipitation experiments and decreased its activation level as observed by a pull-down assay. Moreover, the regulation of Rac1 activity following the double silencing of RhoA and RhoC was abolished by RhoGDIalpha silencing. Our results underscore the competitive binding of RhoGTPases to RhoGDIalpha which regulates their stability and activity. Our observations also imply an indirect regulatory function for the GDP-bound form of the RhoGTPases.

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TRANSCRIPTION FACTOR BINDING SITES IN THE POL GENE INTRAGENIC REGULATORY REGION OF HIV-1 ARE IMPORTANT FOR VIRUS INFECTIVITY

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We have previously identified in the pol gene of HIV-1 a new positive transcriptional regulatory element associated with a nuclease-hypersensitive site (HS7) and containing recognition sites for nuclear proteins (Van Lint et al. (1994), J. Virol. 68:2632-2648). We have next further physically characterized each binding site and have shown that the transcription factors Oct-1, Oct-2, PU.1, Sp1 and Sp3 interact in vitro with the pol region. Chromatin immunoprecipitation assays using HIV-infected cell lines demonstrated that Sp1, Sp3, Oct1 and PU.1 are recruited to the HS7 region in vivo. For each site, we have identified mutations abolishing factor binding to their cognate DNA sequences without altering the underlying amino acid sequence of the integrase. By transient transfection assays, we have demonstrated the involvement of the pol binding sites in the transcriptional enhancing activity of the intragenic region. Our functional results with multimerized wild-type and mutated pol binding sites separately have demonstrated that the PU.1, Sp1, Sp3 and Oct-1 transcription factors regulate the transcriptional activity of a heterologous promoter through their respective HS7 binding sites. Finally, we have investigated the physiological role of the HS7 binding sites in HIV-1 replication and have shown that these sites are important for viral infectivity (Goffin et al. (2005), Nucleic Acids Res. 33:4285-4310). Current studies are examining the role of AP-1 binding sites located upstream of the HS7 region in the enhancer activity and in the viral replication cycle.

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HDAC INHIBITORS AND NF-kappaB INDUCERS COOPERATE TO REACTIVATE LATENT HIV-1 PROVIRUSES IN CELLULAR RESERVOIRS

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Activation of HIV-1 gene expression in latently-infected cells combined with an effective ART has been proposed as an adjuvant therapy that could lead to the reduction of the latently infected reservoirs. We have identified a new regulatory link between protein acetylation and activation of the NF-kappaB signaling pathway by demonstrating a strong synergistic activation of HIV-1 promoter activity by HDAC inhibitors [HDACi] (such as TSA, NaBut, VPA, SAHA, MS275) and inducers of NF-kappaB (such as TNFalpha, PMA, IL-1). This HDACi/NF-kappaB inducer synergism was observed in transient transfection assays for LTRs from subtypes A through G of the HIV-1 major group. Mechanistically, HDACi prolonged the induction of the IkappaBalpha kinase activity, as previously reported by our laboratory for the combination TSA+TNFalpha (Quivy et al. (2002) J. Virol.; Adam et al. (2003), Mol. Cell. Biol.). In latently HIV-infected cell lines, we demonstrated the synergistic activation of HIV-1 transcription and virus production by a HDACi combined with a NF-kappaB inducer. Importantly, our results indicated that HDACi were able to reactivate HIV-1 expression in ex vivo cultures of CD8-depleted PBMCs isolated from aviremic HIV-infected individuals under ART. In similar reactivation assays, we are currently testing the potential synergistic activation of HIV-1 expression by various combinations of HDACi and NF-kappaB inducers.

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DIFFERENTIAL TRANSCRIPTIONAL HIF-1 DEPENDANT RESPONSES BETWEEN HYPOXIA AND IGF-1 IN HEPG2 CELLS

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Abstract Hypoxia induces the expression of a number of genes which are important for the cellular and tissular adaptation to low oxygen conditions. This response is mainly mediated through hypoxia-inducible factor 1 (HIF-1). This transcription factor, composed of two subunits, HIF-1 alpha which is oxygen sensitive and its heterodimeric partner HIF-1 beta, is essential to maintain oxygen homeostasis at the cellular level but it also plays a crucial role in tumor progression, invasion and metastasis. Indeed, HIF-1 alpha is detected not only in the hypoxic center of a solid tumor, but also in the invasive normoxic edges. Growth factors such as IGF-1, EGF, PDGF,… which act mainly through the PI3k pathway, have been shown to enhance the abundance of the HIF-1 alpha subunit and the subsequent activation of HIF-1 in normoxic condition. However, the main mechanism in this induction is an increase in HIF-1 alpha mRNA translation, while hypoxia leads to HIF-1 alpha accumulation through an inhibition of its degradation. Although these pathways are well known, there is currently no data available on a possible specificity of the transcriptional response mediated by HIF-1 according to the type of stimulation.

In order to identify HIF-1 target genes in different conditions of cell stimulation (hypoxic condition and IGF-1 stimulation) we are using chromatin immunoprecipitation assays.

In preliminary studies, we have optimized IGF-1 stimulation conditions (time and concentration) for our cell model (HepG2). We have shown that a stimulation of 6 hours at 100 nM IGF-1 is sufficient to increase HIF-1 alpha abundance and to induce its nuclear translocation. We then studied the effect of some HIF-1 target gene mRNA level by real time PCR. We have shown that the vascular endothelial growth factor (VEGF) mRNA was increased more than 2 fold, as expected, by 16 hours of hypoxia but not by the IGF-1 stimulation. These results were confirmed for some other HIF-1 target genes (CAIX, aldolase,…) but not for all (NMP-1). Using a fast chromatin immunoprecipitation protocol, we are now studying the effect of hypoxia and IGF-1 on the binding of HIF-1 to the HRE localized in the promoter of these same HIF-1 target genes.

This study will help to understand the molecular mechanisms behind these differential transcriptional responses induced by two distinct stimulations activating the same transcription factor via two specific pathways.

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STUDY OF THE ROLE OF HISTONE DEACETYLASES IN MYOFIBROBLASTIC DIFFERENCIATION

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Myofibroblasts (MFs) are usually described as cells characterized by intermediate morphological and biochemical characteristics between those featured by fibroblasts and smooth muscle cells. They participate both in physiologic processes, such as wound healing, and in pathologic conditions, such as chronic inflammation and cancer. In the latter situation, MFs may play a significant role in the remodeling of cancer-associated stroma (reactive stroma), which may favor the progression of several primary human malignancies, including colon cancer. The differenciation of fibroblasts into MFs can be induced by cytokines, amongst which Transforming Growth Factor-beta 1 has been shown to be the prototypic example.

Histone deacetylases (HDACs) constitute a large family of enzymes that regulate gene transcription by modifying the acetylation level of nucleosomal histones and nonhistone proteins, including several transcription factors and tumor suppressors. Global inhibition of histone deacetylase activity by HDAC inhibitors, such as trichostatin A (TSA), suberoxylanide hydroxamic acid (SAHA), has been shown to inhibit tumor growth in vivo in several animal models. HDAC inhibition may affect both the tumor cells and the angiogenic process associated with the tumor. Recent reports have indicated that TSA prevents TGF- beta 1-induced fibrogenesis (synthesis of procollagens alpha (I) and alpha 1(III) and Smooth Muscle alpha -Actin [alpha -SMA]) in cultured rat skin fibroblasts, suggesting that HDACs may regulate MF differenciation.

In this context, we are currently pursuing studies aimed at identifiying the HDAC(s) specifically involved in the regulation of MF differenciation. We have first observed that, in primary human skin fibroblasts, TSA completely abrogates TGF- beta 1-induced overexpression of alpha -SMA, a hallmark of MF differenciation, both at the transcript and protein levels. We are now applying a gene silencing strategy using small interfering RNAs efficiently targeting 8 HDACs (HDAC1 through HDAC8) to investigate which HDAC(s) are most relevant to the control of MF differenciation (initially assessed by the induction of alpha -SMA by TGF- beta 1). We have reproducibly found that TGF- beta 1-induced alpha-SMA overpression is prevented in fibroblasts transfected with siRNAs targeting specifically one of the HDACs investigated. We intend to test whether silencing of this HDAC also affects fibrogenesis and to investigate how HDACs may regulate TGF- beta 1 signaling.

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IDENTIFICATION OF EPIGENETICALLY SILENCED GENES IN TUMOR ENDOTHELIAL CELLS

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Tumor angiogenesis requires intricate regulation of gene expression in endothelial cells (EC). We recently showed that DNA methyltransferase (DNMT)- and histone deacetylase (HDAC) inhibitors directly repress EC growth and tumor angiogenesis, suggesting that epigenetic modifications mediated by DNMTs and HDACs are involved in regulation of EC gene expression during tumor angiogenesis. To understand the mechanisms behind the epigenetic regulation of tumor angiogenesis, we used microarray analysis to perform a comprehensive screen to identify genes downregulated in tumor-conditioned versus quiescent EC, and re-expressed by 5-aza-2'-deoxycytidine and trichostatin A. Among the 81 genes identified, 77% harboured a promoter CpG island. Validation of mRNA levels of a subset of genes confirmed significant downregulation in tumor-conditioned EC and reactivation by treatment with a combination of 5-aza-2'-deoxycytidine and trichostatin A, as well as by both compounds separately. Silencing of these genes in tumor-conditioned EC correlated with promoter histone H3 deacetylation and loss of H3 lysine 4 methylation, however did not involve DNA methylation of promoter CpG islands. Functional validation by RNA interference revealed that clusterin, fibrillin 1 and quiescin Q6 are negative regulators of EC growth and angiogenesis. In summary, our data identify novel angiogenesis suppressing genes which become silenced in tumor-conditioned EC in association with promoter histone modifications and reactivated by DNMT- and HDAC inhibitors through reversal of these epigenetic modifications, providing a mechanism for epigenetic regulation of tumor angiogenesis.

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RECRUITMENT OF P-TEFB (CDK9-CyclinT) TO CHROMATIN BY THE CAP-METHYL TRANSFERASE COUPLES mRNA CAPPING TO RNA POLYMERASE II ELONGATION

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Capping of nascent pre-mRNAs is thought to be a prerequisite for productive elongation and associated Serine 2 phosphorylation of the C-terminal domain (CTD) of RNA polymerase II. The mechanism mediating this link is unknown but is likely to include the capping machinery and P-TEPb.

We report that the fission yeast P-TEFb (Cdk9-Pch1) forms a complex with the cap methyl transferase Pcm1 and these proteins colocalise on chromatin. Ablation of Cdk9 function through chemical genetics causes growth arrest and abolishes Serine 2 phosphorylation on the RNA Polymerase II CTD. Strikingly, depletion of Pcm1 also leads to a dramatic decrease of phospho-Serine 2. Chromatin immunoprecipitations show a severe decrease of chromatin bound Cdk9-Pch1 when Pcm1 is depleted. On the contrary, Cdk9 is not required for association of Pcm1 with chromatin. Furthermore compromising Cdk9 activity leads to a promoter proximal RNA polymerase II stalling and sensitivity to 6-azauracil, reflecting elongation defects.

The in vivo data presented here strongly support the existence of a molecular mechanism where the cap-methyltransferase recruits P-TEFb to chromatin therefore ensuring that only properly capped transcripts are elongated.

Ref: A. Guiguen, J. Soutourina, M. Dewez, L. Tafforeau, M. Dieu, M. Raes, J. Vandenhaute, M. Werner and D. Hermand, The EMBO Journal (in press)

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DECIPHERING BCL-3 DEGRADATION THROUGH IDENTIFICATION OF ITS INTERACTING PARTNERS

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The oncoprotein BCL-3 is a member of the IkappaB family of NF-kappaB inhibitors and was originally identified through molecular cloning of the breakpoint of the t(14;19) chromosome translocation from a subset of human B cell chronic lymphocytic leukemias. BCL-3 is phosphorylated by GSK3 and subsequently degraded through the proteasome pathway. To further gain insight into the molecular mechanisms underlying BCL-3 degradation, we performed yeast two-hybrid experiments using BCL-3 as bait and identified some interacting partners, including PSMB1 and SUMO1. Association of BCL-3 with PSMB1 is enhanced by GSK3-mediated phosphorylation of this oncoprotein. Moreover, degradation of BCL-3 through the GSK3-dependent pathway requires binding to the NF-kappaB proteins p50 and p52. Finally, a cytoplasmic mutant of BCL-3 that is still subject to GSK3-mediated phosphorylation is also polyubiquitinated. Ongoing experiments are carried out to identify the targeted residues of BCL-3 required for degradation. Therefore, our results provide a mechanism by which expression levels of the oncoprotein BCL-3 are regulated and add additional insights into the pathways underlying deregulated NF-kappaB activation in haematological and solid cancers.

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THE LOSS OF E2 INDUCED APOPTOSIS IS ASSOCIATED WITH SUPPRESSION OF DAPK PROMOTER METHYLATION IN HPV-TRANSFORMED KERATINOCYTES.

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Cervical carcinomas are most frequently associated with infection by high-risk human papillomaviruses (HPV). The viral E2 protein is a transcription factor, which regulates the expression of viral oncogens and replication of the viral DNA. E2 protein binds to the specific sites on long control region (LCR) of the HPV genome and represses expression of E6/E7 proteins.

It is well known, that overexpression of E2 cause the apoptosis of transfected cells. We have transfected the cervical carcinoma SiHA cell line with E2 containing plasmid and generated stably expressing cell lines. We found that overexpression of E2 leads to the complete demethylation of the promoters of Death-Associated Protein Kinase (DAPK) and HPV LCR (long control region), whereas promoter methylation of MGMT and TIMP3 remained unchanged. As a consequence of this demethylation, expression of DAPK was restored in transfected cells. Probably, these changes induce the apoptosis in transfected cells through DAPK-dependent mechanism.

Interestedly, that after 16 passages of transfected cells the methylation of DAPK promoter was completely restored. The cells do not show the apoptosis, but they proliferate more slowly in comparison to control cell line transfected with empty vector. We are trying now to understand the molecular mechanism of adaptation of transfected cells to apoptosis.

Demethylation of LCR in transfected cells caused the inhibition of expression of E6 protein probably due to binding of E2 transcription factor to the unmethylated LCR and blocking the transcription from this promoter. Surprisingly, expression of E7 protein remained unchanged. It is well accepted that both E6 and E7 proteins are translated from the same transcript, but our results suggest that transcription of E7 protein may start also from alternative promoter.

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MOUSE PAI-1 PROMOTES PLACENTATION BY INCREASING FOETAL AND MATERNAL ANGIOGENESIS

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Murine placentation is associated with trophoblast cells invasion of the maternal endometrium and extensive maternal and foetal angiogenesis. Both processes involve proteases-dependent extracellular matrix remodeling. Among the protease inhibitors, plasminogen activator inhibitor-1 (PAI-1) is transiently produced by spongiotrophoblasts and trophoblast giant cells at days 10.5-11.5 day post-coitum (dpc). PAI-1 is an angiogenic molecule that promotes tumoral and choroïdal neovascularization. We have therefore analysed the consequence of PAI-1 deficiency on murine placentation and pregnancy outcome. We measured by quantitative computer-assisted image analysis, the relative thickness of the labyrinth, decidua and spongiotrophoblast at day 10.5, 12.5 and 14.5 dpc in PAI-1 deficient and wild type mice. At 10.5 and 12.5 dpc, an abnormal placental morphology was observed in both labyrinth and spongiotrophoblast layers in PAI-1-/- mice. Lack of PAI-1 resulted in a transient decreased maternal and fetal vascularization of the placenta that caused (1) an enhancement in the decidua/labyrinth and labyrinth/spongiotrophoblast thickness ratios, (2) a significant increase of trophoblast density. Normalization of placental morphology occurred by day 14.5 dpc in PAI-1 deficient mice. In conclusion, despite a transient PAI-1 requirement for optimal placental angiogenesis, this gene does not appear to be essential for trophoblast invasion and placentation.

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OSTEOPONTIN EXPRESSION IS REGULATED BY HISTONE DEACETYLASE 4 IN U87-MG GLIOMA CELL LINE

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Malignant gliomas are brain neoplasms that are highly proliferative and angiogenic and are locally very invasive. The median survival of patients with malignant glioma is less than 1 year, and only 2% survive beyond 3 years. A better understanding of glioma biology to derive effective therapeutics for this incurable neoplasm is indicated clearly. Osteopontin (OPN) is a multifunctional extracellular matrix protein involved in tumor growth and metastasis. It is expressed in human glioma tumors and the extent of its expression has been correlated with the malignancy grade. We used specific small interfering RNAs to suppress OPN expression in U87-MG human glioma cell line. These cells were subsequently grafted on the chick chorio-allantoic membrane (CAM) in order to evaluate tumor growth in ovo. Tumors resulting from U87-MG where OPN synthesis was blocked were significantly smaller than control tumors formed from untransfected U87-MG cells (paired t test, $p<0.05$). Histone deacetylase inhibitors are being investigated as unique anticancer drugs because of their anti-proliferative effects on cancer cells. We hypothesized that OPN expression may be under the regulation of histone deacetylases (HDACs) in glioma cancer cells. Indeed, the silencing of different class II HDAC genes revealed that the inhibition of HDAC4 represses OPN expression at the mRNA and protein levels. Moreover, conditioned medium of U87-MG cells where HDAC4 synthesis was blocked contained significantly less OPN than control conditioned medium. Furthermore, we observed using tumor CAM assay that siHDAC4-transfected U87-MG cells generated significantly smaller tumors than untransfected control cells. This is the first demonstration that HDAC4 inhibition down regulates OPN expression and is sufficient to block glioma tumor growth. Ongoing experiments will help elucidating the mechanisms through which HDAC4 potentially exerts its regulation at OPN gene promoter.

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MESENCHYMAL STEM CELL CONTRIBUTION IN CHOROIDAL NEOVASCULARIZATION

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The formation of new capillary blood vessels is implicated in many diseases such as aged-related macular degenerescence (AMD), and may involve different mechanisms including at least angiogenesis and vasculogenesis. Angiogenesis rely on endothelial cell sprouting from existing vessels and vasculogenesis refers to the mobilization and functional participation of bone marrow-derived cells, such as endothelial progenitor cells (EPC), into blood vessels formation. A murine model was used to study BM-derived cell participation to angiogenesis: Choroidal neovascularization (CNV) resulting from the rupture of Bruch's membrane by a laser burn. We focused our work on a subpopulation of BM cells: Mesenchymal stem cells (MSC). These cells can differentiate into multiple cell types including osteoblasts, chondrocytes, adipocytes, and even endothelial cells. Currently, there are several methods available to isolate MSC from bone marrow. In our work, they have been isolated based on their selective adherence to plastic surface, compared to hematopoietic cells.

The putative impact of MSC on angiogenesis was first studied in the aortic ring assay, an ex vivo model of angiogenesis. In the presence of MSC, a pro-angiogenic effect was observed. This stimulation might be mediated by soluble factors, rather than caused by an incorporation of MSC in neovessel wall.

In the in vivo model of CNV, we demonstrated that intravenously injected MSC were specifically recruited into choroidal lesions. MSC were detected in choroidal lesions and not in intact adjacent choroids. We therefore propose to use MSC cells as vehicle to locally deliver an anti-angiogenic agent into choroidal lesions in our CNV model mimicking human AMD.

We are currently generating a lentiviral vector to transduce MSC cells and force them to produce an anti-angiogenic agent.

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S100A4 INHIBITS BREAST CANCER CELLS GROWTH IN THE BONE MICROENVIRONMENT IN VIVO

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Metastatic breast cancer cells exhibit the selective ability to seed and grow in the skeleton. In an attempt to study the osteotropic phenotype of breast cancer cells, our group has previously performed a microarray study on a model of breast cancer bone metastasis consisting of the MDA-MB-231 human cell line and its variant B02 selected for its high capacity to form bone metastases in vivo. Analysis of B02 cells transcriptional profile revealed that S100A4 is the most down-regulated gene (45 fold) in B02 cells when compared to parental MDA-MB-231 cells. S100A4, also known as metastasin, is a small calcium binding protein involved in cell motility, adhesion, proliferation and angiogenesis. In this study, western blotting experiments and RT-PCR confirmed S100A4 downregulation in B02 cells. In order to validate our observations, we performed immunohistochemistry on 4 human primary breast tumors and their matched liver and bone metastases. We found that S100A4 was effectively less expressed in breast cancer bone metastatic lesions than in the corresponding primary tumors and liver metastatic lesions. To further investigate the role of S100A4 in bone metastases development, we restored S100A4 expression in B02 cells through stable transfection (B02-S100A4).No significant difference(s) were observed in terms of in vitro proliferation and attachment between B02-S100A4 and B02 cells. Interestingly, intra-osseous injection of these clones to nude mice demonstrated a significant decrease in tumor growth when compared to original B02 cells that do not express S100A4. Ongoing experiments will help to determine through which mechanism(s) S100A4 expression affects the proliferation of breast cancer cells in the bone microenvironment.

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RANDOM SUBWINDOWS AND EXTREMELY RANDOMIZED TREES FOR AUTOMATIC IMAGE CLASSIFICATION IN CELL BIOLOGY

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Background: With the improvements in biosensors and high-throughput image acquisition technologies, life science laboratories are able to perform an increasing number of experiments that involve the generation of a large amount of images at different imaging modalities/scales. It stresses the need for computer vision methods that automate image classification tasks. Given a set of training images labelled into a finite number of classes by an expert, the goal of an automatic image classification method is to build a model that will be able to predict accurately the class of new, unseen images.

Results: We illustrate the potential of our image classification method in cell biology by directly evaluating it on four public datasets of images related to protein distributions or subcellular localizations, and red-blood cell shapes. Accuracy results are quite good without any

specific pre-processing neither domain knowledge incorporation. Beyond misclassification error rates, the method could highlight discriminative subwindows in images, hence it could be used as an exploratory tool for further biological interpretation. It is implemented as a software in Java and available upon request for evaluation and research purpose.

Conclusion: We foresee the use of this automatic approach as a baseline method and first try in various biological studies that can be formulated as image classification problems.

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ORGANOTYPIC SLICE CULTURES OF RAT CORTEX: MICROSCOPICAL ASSESSMENT AFTER CRYOPRESERVATION

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Organotypic brain slice cultures are widely used as an experimental in vitro model due to the preservation of the basic cellular and connective organization of brain regions in slices. Brain slice tissue cultures provides therefore an easily accessible model for the study of the morphology and degenerative or developmental changes of the central nervous system. These cultures have been increasingly used in molecular biology, electron microscopy, imaging, electrophysiology and immunohistochemical studies. Cryopreservation of brain slices has potential future applications for medical research. It allows viable tissues to be more widely available for testing when and where needed.

In the present study, we investigated possible damage caused by freeze-thawing brain slices.

Cortical brain slices (400µm) of neonatal rats (P5) were made using a vibroslice tissue cutter. Slices were cut and transferred to sterile, transparent ThincertTM inserts. Slices were cultured for 5 days. Other slices were transferred to the same growth medium containing 10% DMSO at 4 C. The freezing process was performed using the Kryo 10-16 controlled rate freezer and stored in -80 C freezer. After cryopreservation slices were thawed and cultured for 5 days in growth medium. For transmission electron microscopic examination (TEM), slices were fixed in 2% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) after 5 days in culture. Tissue was processed using routine EM preparation and examined in a Philips EM 208 TEM operated at 80 kV.

To determine cell viability, staining with 2µM Calcein-AM (Acetoxymethyl) and 5µM propidium iodide (PI) was performed to discriminate between viable and apoptotic or necrotic cells within brain slices. Observations were made using a Zeiss LSM 510 META laser scanning confocal microscope through a 10x / N.A. 0.3 air objective. Excitation of Calcein-AM and PI was respectively at 488nm and 543nm. All measurements were carried out at room temerature (18 C).

After 3 days in culture, slices were firmly attached to the filter membrane. Thawed slices on the other hand, did not attach until day 4, but became firmly attached after 5 days in culture. After 5 days in culture, cell outgrowth at the border of the slices was visualised using phase-contrast microscopy. Thawed slices showed less cell growth.

TEM observations of normal cultured slices showed well preserved structure. Ultrastructural examination of thawed slices showed loss of tissue structure, rupture of cell membranes, spreading of organelles and necrosis. Confocal images confirmed these findings. Thawed slices also showed more PI positive cells compared to normal cultured slices.

Our results showed severe damage in frozen-thawed slices. Cryopreservation has an influence in the tissue structure of brain slices and causes tissue damage. Optimalisation of tissue culture techniques and cryopreservation are being discussed.

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PROTEIN PHOSPHATASE 2A CONTROLS THE SUBCELLULAR DISTRIBUTION AND BIOLOGICAL ACTIVITY OF CLASS IIA HISTONE DEACETYLASES

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Class IIa HDACs (HDAC4, -5, -7 and -9) possess an N-terminal adapter domain which is essential for their regulation and biological function. Several canonical binding motifs for 14-3-3 proteins are present in the N-terminal adapter domain of class IIa HDACs. When phosphorylated on specific serine residues, these consensus motifs recruit 14-3-3 proteins. This interaction overcomes the repressor activity of class IIa HDACs by eliciting their sequestration in the cytoplasm and making them unavailable for their cognate transcription factors and corepressors. Association with 14-3-3 proteins thus confers phosphorylation-dependent regulation and signal-responsiveness to class IIa HDACs.

Class IIa HDACs act as transcriptional modulators of specific genetic programs associated with several key developmental processes. Because regulation of class IIa HDAC phosphorylation provides the opportunity to control these developmental processes, a great deal of effort has been invested in identifying the kinases targeting class IIa HDAC 14-3-3 motifs. It is logical to envision the phosphorylation-dependent regulation of class IIa HDACs as a reversible mechanism. Surprisingly, the identity of such a putative activating class IIa HDAC phosphatase remains unknown. We report here that protein phosphatase 2A (PP2A) is responsible for dephosphorylating the 14-3-3 motifs of class IIa HDACs, thereby regulating their subcellular localization and repressor activity. The functional consequences of these findings are illustrated for the class IIa member HDAC7. In T-cells, inhibition of PP2A leads to the expression of the HDAC7-repressed gene Nur77 with consequent induction of apoptosis. Similarly, siRNA-mediated silencing of PP2A in human endothelial cells recapitulates the effects of HDAC7-deficiency, i.e., derepression of the MMP10 promoter and impaired in vitro angiogenic activity. These results establish PP2A as an essential element in the regulation of class IIa HDACs and unravel an additional mechanism by which the cell can modulate the activity of these key enzymes.

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REGULATION OF CXCL8/IL-8 EXPRESSION BY ZONULA OCCLUDENS-1 IN HUMAN BREAST CANCER CELLS

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Accumulating data now suggest that ZO-1 could be implicated in the regulation of tumor promoting genes, once delocalized from tight junctions. Because of their major implication in different steps of tumor progression, we investigated here the influence of ZO-1 on chemokines expression in breast cancer cells. Using GeneArray analysis to compare chemokine expression in breast tumor cells transfected with a siRNA against ZO-1, we identified CXCL-8/IL-8 as a major potential target of ZO-1 signaling. We observed that the most regulated chemokine in BT549 cells transfected with a ZO-1 siRNA was CXCL8/IL-8. We first demonstrated that CXCL8/IL-8 expression correlates with a relocalization of ZO-1 in breast cancer cell lines. Indeed, CXCL8/IL-8 is overexpressed only in invasive breast cancer cells that display a rather cytoplasmic localization of ZO-1. Non-invasive cell lines displaying a membrane-associated staining of ZO-1 do not express CXCL8/IL-8. Moreover, CXCL8/IL-8 is downregulated in invasive BT549 cells transfected with 3 different ZO-1 siRNA and overexpressed in non invasive BT20 and SKBR3 cells transfected with vectors expressing full length ZO-1 or with a NH2-terminal fragment of ZO-1 comprising the PDZ domains 1-3. We also showed an activation of the CXCL8/IL-8 promoter by ZO-1. Finally, we demonstrated that this regulation of CXCL8/IL-8 by ZO-1 is independent of the -catenin pathway. Our results thus clearly demonstrated an implication of ZO-1 in CXCL8/IL-8 regulation. Because of the major implications of CXCL8/IL-8 in tumor invasion, such a regulation could play an important role in breast cancer progression.

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CHEMOTHERAPEUTIC AGENTS INDUCE THE EXPRESSION OF A NEW VEGF-A ISOFORM (VEGF111) BIOLOGICALLY ACTIVE AND RESISTANT TO PROTEOLYTIC DEGRADATION

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Vascular endothelial growth factor A (VEGF) stimulates vascular endothelial cells survival, proliferation, migration and differentiation, and promotes angiogenesis. Its gene contains eight exons. Various isoforms have been described, all encoded by exons 1 to 5 and differing in the alternative splicing of subsequent exons. Exon 5 encodes a sequence containing the main site of proteolytic cleavage by plasmin and proteases, resulting in VEGF-A degradation.

Chemotherapeutic agents (camptothecin, doxorubicin, mimosin, mitomycin C, taxol and vincristine) induce the expression in a number of human cell lines of a new VEGF isoform (GenBank ID: DQ229900) encoded by exons 1 to 4 and 8. This new variant, named VEGF111 according to the current nomenclature, was not detected in a large array of normal human tissues. Recombinant VEGF111 added to human umbilical and porcine aortic endothelial cells expressing the VEGF receptor-2 (VEGF-R2) induces its phosphorylation, triggers MAPK pathways and intracellular calcium transients. It promotes endothelial cells multiplication, formation of vascular structures in ES embryonic bodies in vitro and angiogenesis in vivo. Contrasting to VEGF 165 and VEGF121, VEGF111 is resistant to proteolysis by plasmin and fluids collected from chronic wounds.

The splicing out of VEGF exons 5 to 7 upon treatment with camptothecin involves ATM-p53 connection as indicated by (1) the inhibition of the process by caffeine, (2) its induction in AT5BIVA cells with forced expression of wtATM, as compared to the parental ATM-deficient cells, (3), its inhibition in HCT116 cells deficient in p53 as compared to the parental p53-proficient cells. Inhibition of the JNK, p38 pathways and protein phosphatase-1 reduced VEGF111 expression induced by camptothecin.

Further work is needed to test (1) the expression of VEGF111 in human and animals cancers and, if detected, its diagnostic and prognostic value, (2) its induction by X-rays and chemotherapy in vivo, (3) the effect of inhibition of its expression or activity in tumorigenesis and cancer therapy, (4) the efficiency of VEGF111 as angiogenesis promoting agent in pathologies with defective vascularization.

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THE TRANSCRIPTIONAL REPRESSOR NIPP1 IS A NOVEL PLAYER IN EZH2-MEDIATED GENE SILENCING

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NIPP1, encoded by the PPP1R8 gene, is a ubiquitously expressed nuclear scaffold protein that interacts directly with a protein kinase (MELK), a protein phosphatase (PP1), pre-mRNA splicing factors (SAP155 and CDC5L) and two polycomb proteins (EED and EZH2). Consistent with the diversity of its ligands, we have found that NIPP1 has a role in pre-mRNA splicing as well as in regulation of transcription and that NIPP1 is essential for early embryonic development and cell proliferation.

MELK and EZH2, two ligands that interacts with the Fork-head- associated (FHA) domain of NIPP1, are frequently overexpressed in many types of cancers and has been implicated in keeping stem cells dedifferentiated. The histone methyltransferase EZH2 is a core component of the Polycomb Repressive Complex 2 (PRC2) and is a newly identified oncogen that promotes the late-stage development of cancer, including hormone-refractory, metastatic prostate cancer. It acts by the repression of differentiation and tumor suppressor genes via trimethylation of histone H3 on Lys27 and recruitment of DNA methyltransferases. Studies on NIPP1-deficient cells disclose a widespread and essential role of NIPP1 in the trimethylation of H3K27 by EZH2, not only in the onset of trimethylation during embryonic development, but also in the maintenance of this repressive mark in proliferating cells. Consistent with this notion, EZH2 and NIPP1 silence a common set of genes, as revealed by gene-expression profiling, and NIPP1 is associated with genomic regions that are enriched in Polycomb targets. Interestingly, the MSMB gene, encoding the tumor suppressor PSP94, belongs to the NIPP1/EZH2 targets.

PSP94, for prostatic secretory protein of 94 amino acids, is secreted by the prostate gland and functions as a suppressor of tumor growth and metastasis. The expression of PSP94 is lost in advanced, hormone-refractory prostate cancer and this correlates with an increased expression of EZH2 and NIPP1, and an increased level of trimethylation on H3K27 of the MSMB gene, compared to hormone-dependent prostate cancer cells. Chromatin immunoprecipitation experiments confirmed an association of EZH2 and NIPP1 with the MSMB gene. Furthermore, the RNAi-mediated knockdown of EZH2 and NIPP1 resulted in a loss of H3K27 trimethylation of the MSMB gene.

In conclusion, the identification of MSMB as an NIPP1/EZH2 target gene can explain why the expression of this tumor suppressor gene is lost in advanced stages of prostate cancer. We suggest that the increased expression of NIPP1/EZH2 in metastatic prostate cancer results in H3K27 trimethylation of the MSMB gene.

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HISTON DEACETYLASES IN HEPATIC STELLATE CELLS: A PRELIMINARY STUDY

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Introduction: Transdifferentiation of hepatic stellate cells (HSC) to myofibroblastic cells is a central event in liver fibrogenesis. Understanding molecular mechanisms that underlie this cellular event provides pivotal insights into development of new therapeutic modalities for cirrhosis. Stellate cell activation by liver injury leads to a phenotypic transdifferentiation characterized by loss of vitamin A and extensive production of extracellular matrix. This process can be mimicked in vitro by culturing freshly isolated hepatic stellate cells. The use of the histone deacetylase inhibitor (HDAC-I) Trichostatin A (TSA) in these cultures has shown that histone deacetylases might play a role in the pathogenesis of liver fibrosis. Short-term treatment of rat HSC with TSA decreases the transcription of alpha smooth muscle actin (αSMA) and collagen III and influences actin filament formation. In this study we want to address the presence, activity and importance of the different HDACs during mouse HSC transdifferentiation

Material and methods: To investigate the possibility of liver- or HSC-specific HDACs, mRNA levels of HDACs were determined in isolated HSC as well as in liver, kidney, brain, pancreas, heart and muscle from Balb/c mice by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). This technique was also used to study the HDAC expression profile during HSC activation in vitro. Furthermore, we analyzed the sensitivity of different markers for activated stellate cells for different HDAC-Is. We currently focus on valproic acid because of its stability and its specificity.

Results: RT-qPCR showed neither liver- nor HSC-specific HDAC mRNAs. The HDAC mRNA profile during transdifferentiation of freshly isolated hepatic stellate cells in culture was not altered. Transcription of several stellate cell transdifferentiation markers is inhibited by long term treatment of different HDAC-Is such as smooth muscle alfa-actin (Acta2), smooth muscle myosin (SMMy1), lysyl oxidase (Lox) and secreted phosphoprotein 1 (Spp1).

Conclusions: We have confirmed in mouse HSC cultures that different HDAC-Is can influence the transdifferentiation from quiescent to activated HSCs. Several markers have been identified that can be used to monitor this process in future RNA interference experiments. This will enable us to identify the HDAC(s) and HDAC complexes that are involved in the activation of HSC in vitro but also in vivo.

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ROLE OF THE LATERAL MESODERM AND FGF SIGNALING IN THE FORMATION OF THE VENTRAL PANCREATIC BUD IN ZEBRAFISH

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It has been known for a long time that, in mammals, mesenchyme-epithelium interactions play important roles for the development of the pancreas. However, no data has been reported so far on the importance of such interactions in lower vertebrates as in amphibians or in fish. In this study, we show that the lateral plate mesoderm controls the development of the pancreatic epithelium in zebrafish embryos and that fgf signaling plays a crucial role in these interactions.

In mammals, fgf10 and isl1 are markers of the pancreatic mesenchyme. By performing in situ hybridization on zebrafish embryos using fgf10 and isl1, we detected expression of these two genes in the lateral mesoderm juxtaposed to the ventral pancreatic anlagen. However, no expression of these two markers was found near the dorsal pancreatic bud. In order to determine the function of fgf10 (and other fgfs) on the ventral pancreatic bud, zebrafish embryos were treated with the FGF receptor inhibitor SU5402 just prior to ventral bud specification. The pancreatic exocrine tissue, which only derives from the ventral bud, was severely disrupted in the treated embryos while the dorsal pancreatic bud was not affected at all. Analysis of several early pancreatic markers revealed that the expression of ptf1a was undetectable in treated embryos. Expression of several markers of the adjacent mesoderm was concomitantly lost. We identified fgf24 as being expressed in the lateral plate mesoderm as fgf10. Furthermore, knock-down of both fgf24 and fgf10 completely blocked the specification and development of the ventral bud.

In conclusion, our data show that, in zebrafish, the lateral mesoderm juxtaposed to the ventral pancreatic epithelium express FGF signals required for the activation of the ptf1a gene and thus for the proper development of the ventral pancreatic bud.

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REGULATION BY ANDROGENS OF THE EXPRESSION OF THE EGF RECEPTOR FAMILY MEMBERS IN PROSTATE CANCER CELLS

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After an initial positive response to anti-androgen treatment, prostate cancer (PCa) cells usually become hormone-refractory in spite of their persistent expression of the androgen receptor (AR). Overexpression of tyrosine kinase receptors in androgen-deprived PCa cells may be responsible for AR activation and growth of androgen-deprived tumours. Our goal is to understand the control of the expression of the EGF receptor (EGFR) family members by androgens in PCa. Hormone response was compared in hormone-sensitive LNCaP and hormone-insensitive DU145 PCa cell lines. Dihydrotestosterone (DHT) modulates EGFR and erbB2 transcript and protein levels only in LNCaP cells. DHT treatment of cells cultured in steroid-deprived medium increases EGFR mRNA and protein levels, while it inhibit erbB2 mRNA and protein levels. ErbB3 is not an androgen-responsive gene. These effects are time and dose dependant and are partially inhibit by bicalutamide, an AR antagonist. Inversely, treatment of LNCaP cells cultured in complete medium by siRNA against RA induce a reduce level of EGFR protein and an increase level of erbB2 receptor. In order to understand the mechanisms by which androgens control the expression of EGFR and ERBB2 genes, half-lifes of the corresponding mRNAs and proteins were compared in cells cultured in presence or absence of DHT. No difference in the stability of RNA and protein coding by EGFR and ERBB2 were observed following DHT treatment suggesting a transcriptionnal effect of DHT on EGFR and ERBB2 expression. Indeed, ChIP experiment show recrutement of RNA polymerase II on the promoter of EGFR gene after the hormone stimulation. Moreover, by blocking traduction with cycloheximide before hormone stimulation, the increase of EGFR mRNA is always present while the repression of erbB2 mRNA is lost. These data indicate that DHT through the androgen receptor act directly to stimulate the transcription of EGFR and indirectly on the transcription of erbB2. Future experiments will characterise the functionality of the putative ARE present on the EGFR promoter by EMSA, reporter vector and ChIP assays.

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ZIC1 GENE EXPRESSION IN MESENCHYMAL PROLIFERATIONS IS CONTROLLED BY METHYLATION

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Zic genes belong to a conserved family of zinc finger proteins playing critical roles during embryonic development. The zic1 gene, a member of the Zic family genes, has been suggested that participates in the regulation of cell proliferation of dorsal neural tube during embryonic stage. Here we characterize ZIC1 expression in the desmoid tumors and fibroproliferative states.

Expression arrays revealed the consistent upregulation of the neural transcription factor ZIC1 in primary desmoid cultures when compared to primary fascia cultures. ZIC1 expression was also found in other fibroproliferative disorders, in activated fibroblasts during wound healing, and proliferating skin fibroblast in culture, whereas it is absent in resting fibroblasts. The dynamic ZIC1 expression in mesenchymal proliferations is novel and suggests a proliferative role for ZIC1 in these tissues. We found a potential role of DNA methylation, a well known epigenetic regulator of gene expression, in the dynamic expression of the ZIC1 gene. We show that the 5' flanking region of ZIC1 promoter is unmethylated in desmoid tumor in compare to the adjacent fascia fibroblasts where this region is more methylated. This high level of methylation at the ZIC1 promoter in fascia cells fully corresponds with the lack of ZIC1 expression observed by RT-PCR. Treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza-dC), resulted in a five fold upregulation of ZIC1 expression in fascia cells. To our knowledge, this is the first report describing ZIC1 expression in mesenchymal proliferations and a role for DNA methylation in the control of ZIC1 expression. Recently dynamic zic1 regulation by polycomb factors was found to be essential during stem cell maintenance and differentiation. Our findings indicate methylation regulates zic1 expression postnatally in novel tissue compartments.

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CONTRIBUTION OF MASS SPECTROMETRY (SELDI-TOF-MS) TO THE ANALYSIS OF PROTEINS IN INFLAMMATORY CONDITIONS

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A complex network of interactions between inflammatory cells, peptidic mediators, extracellular matrix and proteases is thought to be involved in the control of asthma-related inflammation. The precise role of many new mediators of proteic nature in the pathophysiology of inflammation remains largely unexplored.

In order to determine which mediators are susceptible to be implicated in asthma progression, we used SELDI-TOF-MS (Surface Enhanced Laser Desorption/Ionisation-Time of Flight-Mass Spectrometry) for the detection of low molecular weight peptides differentially expressed in lung tissue from mice exposed either to allergen or placebo. When studying proteins profiles from placebo or allergen-exposed mice, we identified a group of proteins that were modulated in the lungs from allergen-exposed animals in the acute inflammation protocol. Among those, we point out the protein, HIMF (hypoxia induced mitogenic factor) as being overexpressed in asthma. We also demonstrate that HIMF, a newly described Th2 multipotent cytokine, is upregulated in allergen-induced inflammation by western blot and semi-quantitative RT-PCR. HIMF is an inducible secreted protein expressed in lung tissue and displays mitogenic, antiapoptotic, pro-angiogenic and vasoconstrictive properties. The precise in vivo functions of HIMF are still largely unknown, especially in the lung. We conclude that HIMF, a factor described to be associated with hypoxia, is overexpressed in the context of allergen-induced inflammation.

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LEVONORGESTREL-RELEASING INTRAUTERINE DEVICE INSERTION LEADS TO VESSEL SIZE VARIATION AND FRAGILITY

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Background: Levonorgestrel-releasing intrauterine system (LNG-IUS) is contraceptive system which is also licensed for the treatment of menorrhagia. It is usually inserted to reduce menstrual blood loss, but compliance is often hampered because of the initial troublesome side-effect of breakthrough bleeding which is most commonly seen during 4-6 months after insertion of the LNG-IUS. The objective of the study is to elucidate the mechanism responsible for this paradoxical bleeding by analysing the endometrial vasculature. Methods: The morphology, size, density and maturity of all endometrial vessels were assessed in endometrial biopsies from patients bearing LNG-IUS for a short time (1 to 3 months) or a long time (more than 12 months) exposure. Determination of vessel maturation was determined by a double immunostaining using anti-von Willebrand Factor and anti-alpha Smooth Muscle Actin (alpha-SMA) antibodies. According to alpha-SMA staining intensity, a score (0, 1 or 2) was attributed to blood vessels. Vessel size, number and density were determined quantitatively with a computer-assisted image analysis system. Results: The endometrium impregnated by LNG revealed a significant increase of relative vascular area and an enhanced vessel number when compared to control endometrium. In addition, vessel area varied according to LNG-IUD period use. Indeed, short term LNG group presented smaller vessels as compared to the control group for all vessel categories. The long term LNG group showed larger immature vessels. Conclusions: Levonorgestrel affects blood vessel size, number and maturity in a pattern that changes with duration of exposure.

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STUDY OF CERAMIDE SIGNALING PATHWAYS BY DIFFERENTIAL ANALYSIS OF THE PROTEOME OF CANCER CELLS

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Ceramides appeared to be intracellular second messengers playing a role in the regulation of the cell growth, differentiation, and programmed cell death. Several cytokines and environment stresses including ultraviolet radiation, chemotherapeutic drugs and oxidative stress, induce a rapid rise of intracellular ceramide concentration by neosynthesis or by hydrolysis of sphingomyelin. Ceramide may induce the activation of phosphatases, protein-kinases (PKC,MAP kinases) and transcription factors (NF-kappaB, AP-2). The mechanisms by which ceramides modulate gene expression and protein synthesis are still unknown.

To better understand how these secondary messengers induce their biological effects, we stimulated cancer cells with exogenous long chain ceramides in order to mimic endogenous sphingolipids. Indeed, we observed by mass spectrometry than only long chain ceramides (C16-, C18-, C20-, C22-, C24-ceramides) are present in HCT-116 human colon adenocarcinoma cells. On the contrary to synthetic short chain ceramides (C6-ceramide), the long chain ceramides do not penetrate spontanously into the cell. A strategy to incorporate ceramides with long fatty acid chain was elaborated and checked by tandem mass spectrometry.

Then, we demonstrated by viability tests that exogenously-supplied ceramide are cytotoxic. The absence of toxicity to the concentrations used of dodecane and ethanol was also shown. Moreover, cytochemical staining by Annexin-V-FITC/ propidium iodide and flow cytometry analyses indicate than long chain ceramides induce cell death by necrosis and apoptosis. The cell apoptosis has been confirmed by immunoblotting analysis. Indeed, we observed a activation of caspase-3 and a poly(ADP-ribose) polymerase proteolysis at 4h in HCT116 cells treated by C16-ceramide.

In order to study the role of long chain ceramide in transmission of stress signals, a differential analysis of HCT-116 treated or not by C16-ceramide was realised by two-dimensional differential in-gel electrophoresis (2D-DIGE). Bioinformatic analysis revealed differential expression of forty proteins. Protein identification was performed by MS-MS. Some of these identifications have been confirmed by Western blot. We are currently studying the implication of these proteins in pathways activated by long chain ceramides.

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INSIGHT INTO Hut78 ONCOGENIC POTENTIAL

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Chromosomal rearrangements of the nfkb2 gene have been observed in a number of cases of human cutaneous T cell lymphomas, as well as human B-cell lymphomas and myelomas, suggesting that they may contribute to lymphomagenesis. These rearrangements cluster within the 3'-terminal ankyrin-encoding domain of the nfkb2 gene and lead to the production of C-terminally truncated proteins. Still, the mechanisms underlying the oncogenic potential of such proteins are poorly defined. Hut78 was identified in a cutaneous T-cell lymphoma (CTCL) cell line (Hut78) and is composed of the 665 first amino acids of p100 fused to 3 extra amino acids (serine-alanine-serine). To better understand why Hut-78 is oncogenic, we first assessed its localization by immunofluorecence and noticed that this protein is nuclear. Moreover, through generation of mutants lacking key functional domains of Hut-78, we mapped the regions of this oncoprotein required for nuclear localization. We also generated Hut-78 mutants whose interaction with co-repressors such as HDAC proteins is enhanced. To address Hut-78 oncogenic potential in vivo, we infected NIH3T3 cells with a Hut-78 over-expressing retrovirus and subcutaneously injected these resulting cells in nude mice. We did not observe any tumor formation in these mice, which suggests that additional genetic alterations, yet to identify, are required. Moreover these cells are unable to form foci in vitro and we could not detect any differences in their growth curves, compared to cells infected with the control. In order to determine if the protein Hut78 directly regulates gene expression, we are currently analysing the gene expression profile of over-expressing Hut-78 NIH3T3 cells. Taken together, these results provide further insights into Hut-78 oncogenic potential, and by extension, will help to better understand how mutated IkappaB proteins contribute to deregulated NF-kappaB activity in haematological diseases.

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ESTABLISHMENT OF A TRANSGENIC T CELL-BASED ADOPTIVE IMMUNOTHERAPY MODEL IN EL-4-OVA TUMOR BEARING MICE

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The CD94/NKG2A heterodimer is an MHC class I-specific inhibitory receptor expressed by NK cells, where it has been originally characterized, but also by a small fraction of activated T lymphocytes, predominantly of CD8+ phenotype. Its major physiological role is to induce immune tolerance towards normal self cells. An abnormal upregulation of this receptor has been observed on CD8+ T cells from patients affected by several bacterial and viral infections or bearing various malignancies, suggesting that it could be one of the immunosurveillance escape pathways used by cancer cells. In order to better understand the mechanisms responsible for this NKR upregulation on T cells surface, we analyzed its expression on naive CD8+ T lymphocytes from OT-I/RAG1-/- mice activated by the fibroblasts MEC.B7.SigOVA and treated with PGE2 or LPS, an inducer of COX-2. First, we demonstrated that our in vitro priming system could elicit CD8+ T cells proliferation and differentiation. Then, we showed that only a small percentage of activated CD8+ T cells expressed CD94/NKG2A whereas this fraction increased when PGE2 or LPS was present after the activation period. The observed LPS effect on the expression of this inhibitory receptor on CD8+ T cells surface was dependent on a cell population other than CD8+ T lymphocytes themselves, since it was lost on sorted CD8+ T cells, and it was partially inhibited by indomethacin, a non-specific COXs inhibitor, suggesting the potential involvement of COX-2 and PGE2 synthesis in this process. Finally, we found that CD8+ T cells expressing this receptor after PGE2 treatment had a decreased lytic capacity. These preliminary findings suggest that PGE2 can interfere with the cytolytic activity of CTLs probably by inducing an over-expression of the CD94/NKG2A receptor on their surface.

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MALIGNANT IL6 GENE EXPRESSION IN METASTATIC BREAST CANCER CELLS IS EPIGENETICALLY DETERMINED BY AP1 FRA1 AND NFkB P65

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Weak or strong IL6 transcription in benign MCF-7 or metastatic MDA-MB-231 breast cancer cells was found to strongly depend on the epigenetic settings of the promoter. Cell type specific differences in promoter hypersensitivity to DNase, micrococcal nuclease and restriction enzyme digestion reveal differential nucleosome accessability across the promoter region, including AP1 and NFkB transcription motifs. Interestingly, in unstimulated MDA-MB231 cells, we observe strong basal recruitment of AP1 (fra1, jun) transcription factors and the histone acetylases p300, which can prime IL6 promoter chromatin for permissive transcription. Furthermore, we demonstrate TNF inducible recruitment of the kinase MSK1, the acetylase CBP and the chromatin remodeling factor Brg1 at the IL6 gene promoter, concomitantly with increased H3 phospho-acetylation and reduced H3 dimethylation, which nicely corresponds with maximal recruitment of NFkB p65 and strongly elevated IL6 gene transcription levels. In contrast, in MCF7 cells, lack of basal recruitment of AP1 fra1/jun/p300 may prevent efficient release of histone deacetylase HDAC1 and heterochromatin protein HP1, which prevents recruitment of p65/MSK1/Brg1 at the IL6 gene promoter, resulting in sustained H3 dimethylation, suboptimal H3 phospho-acetylation and silencing of IL6 gene transcription. Interestingly, activated NFkB/MSK1 fails to relieve IL6 promoter silencing in MCF7 cells. However, upon complementation of MCF7 cells with the AP1 member fra1, which is overexpressed in MDA-MB231 cells, chromatin accessability at the proximal promoter and IL6 gene expression is strongly increased. As such, overexpression of AP1 members may promote aggressive and metastatic breast cancer by changing the epigenotype of NFkB target genes.

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DETECTION OF DNA METHYLATION IN INFLAMMATORY BREAST TUMOURS BY A MULTIPLEX NESTED METHYLATION-SPECIFIC PCR

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Introduction. Hypermethylation of gene promoters is a common mechanism of loss of gene function in cancer cells. Thus far, few data on epigenetic alterations in inflammatory breast cancer (IBC) cells are available. Microarray studies have shown that IBC is characterised by a distinct gene expression profile when compared to non-IBC. In this study, we sought to determine whether the profiles of gene hypermethylation differ between IBC and non-IBC. To this purpose, a multiplex nested methylation-specific PCR (MSP) was used, which allows the simultaneous analysis of multiple gene promoters. A total of 10 genes for which evidence exists for involvement in breast carcinogenesis by means of methylation were selected.

Methods. The SLµCut system (MMI) was used for laser capture microdissection of paraffin-embedded tissues. 10-µm-thick sections from 10 patients with IBC and 12 patients with non-IBC were used. Genomic DNA from the microdissected tissue was extracted with the QIAamp DNA Micro Kit (Qiagen) and treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research). The methylation status of the promoter regions of APC, E-cadherin, H-cadherin, thrombospondin, DAPK, FHIT, GSTP1, p16, RASSF1A and BRCA1 were investigated using a multiplex nested MSP method. DNA isolated from normal peripheral lymphocytes from healthy individuals served as a negative methylation control. In vitro methylated DNA served as the positive methylation control. MSP products were analyzed on agarose gel electrophoresis, and were determined to have methylation if a visible band was observed in the methylation reaction.

Results. A minimum tissue area of 6mm2 was microdissected from each sample. The average DNA yield was 762 nanograms. Methylation of the APC gene promoter was observed in 33.3% (4/12) of non-IBC samples and 70% (7 /10) of IBC samples. Methylation of the BRCA1 gene promoter was observed in 16.7% (2/12) of non-IBC samples and 30% (3/10) of IBC samples. Methylation of the DAPK gene promoter was observed in 16.7% (2/12) of non-IBC samples and 10% (1/10) of IBC samples. No methylation of the E-cadherin gene promoter was observed in non-IBC samples or in IBC samples.

Conclusions. Also in this study, the multiplex nested MSP approach, originally developed by Palmisano et al. (2000), was proven to be very suitable for the detection of methylation in samples harbouring small amounts of poor quality DNA. At this moment, the methylation status of the other genes is being determined. Furthermore, the study population will be enlarged.

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SOMATOSTATIN AND SOMATOSTATIN RECEPTOR (SSTR) DISTRIBUTION IN THE ILEUM OF SSTR2 KNOCK-OUT/LACZ KNOCK-IN MICE

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Previous reports showed the involvement of the somatostatin (SOM) receptor 2A (SSTR2A) in the inhibitory effects of SOM in gastrointestinal motility and the inflammatory suppressive effects of SOM at various sites of inflammation. However, our recent results on the expression of multiple SSTR subtypes in the murine ileum and the unchanged expression pattern of SSTR2A during intestinal inflammation, urged to further evaluate the role of SSTR2 in the murine ileum. In the ileum of normal and acutely Schistosoma mansoni-infected SSTR2 knock-out/lacZ knock-in (SSTR2-/-) mice, we studied the distribution of SOM and SSTRs using immunocytochemistry, RT-PCR and quantitative Real Time RT-PCR (qPCR). Concluding the morphological data, no significant changes were detected in the distribution of SOM or SSTRs in the SSTR2-/- ileum compared to the wild-type littermates, either in non-inflamed nor in inflamed conditions. In the non-inflamed SSTR2-/- ileum, SOM was detected in enteric neurons, endocrine epithelial cells and a limited number of CGRP immunoreactive (ir) nerve fibers in the lamina propria. SSTR1, SSTR3 and SSTR4 showed similar distribution patterns as wild type animals, both in inflamed and non-inflamed conditions, with expression on nerve fibers, epithelial cells and mucosal mast cells. No SSTR2A or SSTR5 immunoreactivity (IR) was detected. qPCR analysis revealed no significant changes in the mRNA levels of any of the SSTRs compared to the normal and infected wild-type mice. Intestinal inflammation induced significantly increased ileal SOM mRNA levels in wild-type and SSTR2-/- mice. However, SOM mRNA levels were significantly lower in SSTR2-/- mice than in wild type animals, both in non-inflamed and inflamed conditions. Since the number of endocrine SOM-ir epithelial cells was similar in both mouse strains in both conditions, this reduction is probably due to a decreased number of SOM-expressing enteric neurons in SSTR2-/- mice. These results support our previous data showing the involvement of mainly SSTR1, SSTR3 and SSTR4 in the somatostatinergic inflammatory effects during intestinal schistosomiasis. The present study assumes a possible link between the expression level of SOM in enteric neurons and the presence of SSTR2A in the enteric nervous system.

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THINKING ABOUT LIVING ORGANISMS: THE IMPORTANCE OF EPIGENETICS AS A CONCEPTUAL SCHEME

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The implications of molecular biology for society are immense. Nonetheless, due to its highly specialized technological basis and fast rate developments in experimental and conceptual setup on the one hand and coarse-grained communication channels on the other hand, the societal penetrance of insights and views from this discipline is rather slow. This gap makes 'the perception of molecular biology' a rich study domain for social scientists, historians and philosophers. For example, the new book 'The effects of genetics on contemporary thinking' (edited by A. Fagot-Largeault, S. Rahman, J.M. Torres, Kluwer Academic Publishers, in press) discusses how contemporary genetics is present in and has an influence on domains other than the strict genetical or biological field of knowledge. In this, the editors focus on 'the increasing geneticization of (scientific) thinking'. However, the concept of 'geneticization' should not be read uncritically. In order to give meaning to this concept, it needs to be addressed what contemporary molecular biology stands for and wherein its conceptual and practical approach towards living systems lies. This exercise repeatedly has been done in diverse media as well as in philosophy of biology. In this, the focus on molecular biology as being mainly gene-centric ('a gene for x') and reductionistic ('it's all in the genes') received major attention.

Here, it is argued that such a focus has become outdated and that today 'geneticization' can also be interpreted as 'epi-geneticization'. This conceptual shift is supported by a longer history of researchers and scholars (ranging from the natural philosopher Aristotle to the embryologist Conrad Waddington, who coined the term 'epigenetics' in the late 1940s) envisioning living organisms as dynamic systems, instead of preformed systems. This shift finds further input from experimental research in molecular biology itself, i.e. in the subdiscipline of epigenetics. Epigenetic studies not only provide sophisticated research indicating that not all heritable information leading to the phenotype is inscribed in the DNA base sequence. Epigenetics – as the term indicates – also 'goes beyond' genetics by taking up the challenge of approaching biology in less gene-centric terms. It allows to question the sovereign role of the genome, and to explore if there are multiple contexts of determination at play in the organisation of a biological system. As such, epigenetics is more than just another discipline in molecular biology, as it holds implications for the way traditional concepts (such as 'gene', 'heredity', 'evolution', 'mutation') are understood and for the manner in which ways of thinking about living organisms are communicated to a larger audience.

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NOVEL ASSOCIATION OF SIP1/ZFHX1B WITH NURD AND ITS RELEVANCE FOR MOWAT-WILSON CONGENITAL SYNDROME

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Loss of heterozygosity of ZFHX1B (zinc finger homeobox 1B protein), encoding the transcriptional repressor Smad-interacting protein-1 (SIP1), is implicated in the etiology of Mowat-Wilson Syndrome (MWS). All patients with typical MWS present severe mental retardation and display multiple congenital defects. Analysis of the embryonic phenotypes of Sip1 conventional knockout mice, bearing a drastic truncating mutation comparable to those found in several human patients, revealed that the neurological problems seen in these MWS patients can be ascribed to neural crest defects and early neurogenesis defects. Recently also non-truncating mutations in the ZFHX1B gene in patients presenting a mild form of MWS were identified. The precise mechanisms underlying the aberrant functions of mutant SIP1 protein in patients however, are unknown. Using mass spectrometry analysis we identified subunits of the nucleosome remodeling and histone deacetylase (NuRD) co-repressor complex in affinity-purified Sip1 complexes. Sip1 associates with NuRD through its N-terminal domain, which harbors a NuRD binding motif. Interestingly, this motif is substituted by an unrelated sequence in a recently described MWS patient. We show here that the aberrant human SIP1 protein is unable to recruit NuRD subunits. Moreover, a mutant form of the well-characterized ATPase subunit of NuRD, Mi-2beta, reduces Sip1 ability to repress E-cadherin promoter activity in vitro. XSip1 is a crucial regulator of early neurogenesis in Xenopus. Morpholino-based knock-down of XMi-2beta reduces XSip1-induced Sox2 expression in Xenopus animal cap explants, evidencing also a requirement of Mi-2beta for XSip1-mediated neural induction. Thus, NuRD and Sip1 proteins functionally interact, and defective NuRD recruitment by mutant SIP1 is a potential disease-causing mechanism of MWS. This is the first study providing mechanistic insight into aberrant function of a domain mutant of SIP1 in this syndrome.

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MeCP2 TARGETS THE POLYCOMB GROUP PROTEIN EZH2 TO PROMOTERS

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Polycomb Group (PcG) proteins and DNA methylation are fundamental epigenetic systems involved in gene silencing. Recently we have uncovered a close connection between these two systems: the PcG protein EZH2 can control DNA methylation (1).

Here we show that conversely, CpG methylation can influence EZH2 function through the methyl-CpG-binding protein MECP2. We demonstrate that EZH2 interacts physically with MECP2 in vivo. Chromatin immunoprecipitations indicate that the appearance of EZH2 on promoter sequences coincides with binding of MeCP2 and with underlying CpG methylation Using MeCP2-knockdown cells or a demethylating agent, we show that DNA methylation and MeCP2 are required for promoter occupancy of EZH2 and for H3K27 trimethylation

Our results suggest that MECP2 may act as a “molecular scout” for PcG recruitment to chromatin. They could shed light on the poorly understood mechanisms by which mammalian Polycomb Group proteins are targeted to promoters.

Reference

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GENETIC BACKGROUND-SPECIFIC PROCESSING OF ARTIFICIAL (SUB-)TELOMERES ON A LINEARIZED CHROMOSOMAL VECTOR IN TELOMERASE-POSITIVE MAMMALIAN AND AVIAN CELLS.

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Telomere dysfunction on one chromosome may compromise the integrity and stability of the whole genome. This underscores the importance of comprehending the mutational processes active at the chromosomal ends. We analyzed on a designer chromosome telomeric mutations induced by genetic background switching. The latter was accomplished by microcell-mediated transfer of the chromosome (MMCT) to different rodent and avian telomerase-positive cell lines. We demonstrate that the processing of an existing functional telomere depends on the new genetic background. Besides telomere length adaptation (reduction or elongation), also de novo subtelomeric DNA methylation was detected in some telomerase-positive rodent cell lines and in vivo in mice, suggesting subtelomeric epigenetic mechanisms are directly involved in appropriate telomere processing and function. These epigenetic mechanisms may also underlie telomeric position-effects that result in subtelomeric gene silencing. All modifications are independent of the MMCT procedure per se. Additionally, the protective function of a telomere is not necessarily maintained in a new telomerase-positive genetic background as deduced from the observed chromosomal recombinations. These findings also have important implications for chromosomal vector technology.

The designer chromosome was a circular chromosomal vector (CV) linearized by site-specific de novo telomere formation. Linearization was confirmed by the detection of decreasing CV-specific terminal restriction fragments after treating the genomic DNA with the Bal31 exonuclease. Targeted telomere-associated chromosome fragmentation shows that the linear CV consists of centromeric alphoid-20 sequences flanked by 1p22.1-21.3-derived chromosomal arms each ending with an identical artificial sub-telomere. The mitotic stability of the linear CV had increased when compared to its circular version in mouse ES-cells.