4th SIBBM Seminar

"Frontiers in Molecular Biology"

15th/17th May 2008

www.sibbm.org/seminario2008.php

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4th SIBBM Seminar "Frontiers in Molecular Biology"
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ABSTRACTS OF INVITED SPEAKERS

Small RNAs function as genome defence mechanism and key regulators of gene expression

Giuseppe Macino

Universita' La Sapienza, Roma

In early nineties there were several report in Plants and fungi about an unknown mechanism of gene silencing affecting gene expression in transgenic organisms. Since then much is now known. RNA silencing is a natural mechanism of gene regulation in cells and is used as defence against transposons and viruses. Small double-stranded RNA molecules 20-28 nucleotides long trigger the degradation of target RNA or DNA, thereby reducing specific gene expression. Furthermore in recent years very abundant small RNA molecules called MicroRNA were found in mostly of the eukaryotic cells encoded by endogenous genes. MicroRNA are now considered the most promising regulators of gene expression in normal and pathological tissues. Since then an enormous number of groups joined the field of RNA silencing producing an impressive acceleration to the studies and their possible applications.

Regulatory circuitries controlled by microRNAs in myeloid differentiation

Rosa A., Ballarino M., Fatica A., De Marchis M.L. and Bozzoni I.

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microRNAs are an abundant class of small non-coding RNAs that play important roles in post-transcriptional gene regulation. In animals, they are implicated in an increasingly wide variety of biological processes and their expression appears to be tissue-specific and highly regulated according to the cell's developmental lineage and stage. While thousands of mammalian genes are potentially targeted by miRNAs, the functions of miRNAs in the context of gene networks controlling cell differentiation are not well understood. Because of the wealth of information available about the transcriptional and cellular networks involved in hematopoietic differentiation, the hematopoietic system is ideal for studying the role of miRNAs in cell lineage specification.
In particular, we are interested in the identification of miRNAs involved in human myelopoiesis. We have recently identified a minicircuitry involving miR-223, the myeloid lineage-specific transcription factor C/EBPalpha and the transcription factor NFIA in controlling granulopoiesis (Fazi et al., 2005, Cell 123: 819-31). Subsequently, we identified another regulatory network between the master myeloid transcription factor PU.1, miR-424 and NFIA in regulating human monocytic differentiation. We show that PU.1 activates the transcription of miR-424 that, in turn, stimulates differentiation through translation repression of NFIA (Rosa et al., 2007, PNAS 104:19849-54).
Our data point to a key recurring function of miRNAs in the context of gene regulatory networks involved in hematopoietic lineage differentiation by reinforcing the gene expression program of differentiated cellular states.

MicroRNA control of Nodal signaling

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MicroRNAs are crucial modulators of gene expression, yet their involvement as effectors of growth factor signalling is largely unknown. Ligands of the transforming growth factor-b superfamily are essential for development and adult tissue homeostasis. In early Xenopus embryos, signalling by the transforming growth factor-b ligand Nodal is crucial for the dorsal induction of the Spemann's organizer. Here we report that Xenopus laevis microRNAs miR-15 and miR-16 restrict the size of the organizer by targeting the Nodal type II receptor Acvr2a. Endogenous miR-15 and miR-16 are ventrally enriched as they are negatively regulated by the dorsal Wnt/b-catenin pathway. These findings exemplify the relevance of microRNAs as regulators of early embryonic patterning acting at the crossroads of fundamental signalling cascades.
MicroRNAs (miRNAs) represent a new class of non-coding regulatory endogenous pool of miRNAs expressed in early Xenopus embryos.

Many faces of the RNA binding protein KSRP

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The interaction of different protein factors with target RNA sequences is essential for the intricate series of events that determine post-transcriptional control of gene expression. KSRP is a single strand RNA-binding protein that interacts with distinct RNA sequences and is involved in different steps of post-transcriptional RNA life from splicing to transport, from cellular localization to decay. Many studies from our and other laboratories demonstrated that KSRP binds to inherently unstable mRNAs and target them for rapid degradation recruiting the ribonucleolytic enzymes. Now, we have found that KSRP is also implicated in an additional and unexpected step of post-transcriptional regulation of gene expression. KSRP is a component of both Drosha and Dicer complexes and binds with a high affinity to the terminal loop of a subset of miRNA precursors, critically regulates their maturation and, in turn, the ability of the mature forms to appropriately inhibit expression of target mRNAs. These findings reveal an unexpected mechanism that links KSRP to the machinery regulating maturation of a cohort of miRNAs, that, coupled to its role in promoting mRNA decay, serves to integrate specific regulatory programs of protein expression.

This work has been supported by AIRC, ISS Italia-USA, e CIPE 2007 Regione Liguria.

The role of the Epigenome in gene regulation

Valerio Orlando

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Conrad Waddinton (1905-1975) is credited with coining the term epigenetics in 1942 as the "branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being". However, the debate about the origin of the living and how its forms depend on its biological experience dates back to Aristotle who believed in epigenesis. The debate continues today, to understand to which extent we are preprogrammed versus the role of the environment in shaping us.

More recently, the field of epigenetics has emerged to bridge the gap between nature and nurture. In fact, in the 21st century epigenetics defines the study of of heritable changes in genome function that occur without changes in the DNA sequence.

As a matter of fact, short after the completion of the genome projects it became obvious that the simple, monodimensional information contained in the DNA is not sufficient to anticipate how genome works. From chemical modification of DNA and chromatin components, up to spatial and dynamic organization of genes and regulatory elements in the nucleus, all levels of chromosome organization appear to contribute to provide additional levels of information that matter for the stability and heritability of transcription programs. These parameters help to understand the molecular basis of fundamental aspects developmental programs and inheritance phenomena that do not follow the mendelian rules.

The lecture will focus on the relationship between the monodimensional nature of genetic information (DNA) with multidimensional parameters that contribute to gene regulation (the Epigenome).

The Nucleosome Remodeling ATPase ISWI is Regulated by poly-ADP-ribosylation

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ATP-dependent nucleosome remodeling enzymes and covalent modifiers of chromatin set the functional state of chromatin. However, how these enzymatic activities are coordinated in the nucleus is largely unknown. We found that the evolutionary conserved nucleosome-remodeling ATPase ISWI and the poly-ADP-ribose polymerase PARP genetically interact. We present in vitro and in vivo evidence showing that ISWI is target of poly-ADP-ribosylation. Remarkably, the poly-ADP-ribosylation of ISWI causes a reduction of its chromatin binding and function. We propose that one of the in vivo functions of PARP is to counteract ISWI chromatin remodeling activity. Consistent with this model, we found that ISWI and PARP localize to distinct chromatin domains. Our work suggests that ISWI is a physiological target of PARP and that poly-ADP-ribosylation can be a new important posttranslational modification regulating the activity of ATP-dependent nucleosome remodelers.

Lysine-specific Histone Demethylase (LSD1): Oxidative Chemistry for Chromatin Remodelling

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In the last years, we have studied a nuclear protein complex formed by the association of histone deacetylase 1, a co-repressor protein CoREST, and a protein formerly known as KIAA0601 or BHC110. Our group and the group of Y. Shi (Harvard University) have discovered that this protein is a lysine-specific histone demethylase (now called Lysine Specific Demethylase 1; LSD1) that specifically acts on Lys4 of histone H3.
Three years after this discovery, LSD1 remains at the forefront of chromatin research. Its demethylase activity on Lys4 of histone H3 supports its role in gene repression. Data from our and other laboratories indicate that the complex formed by LSD1 with histone deacetylases 1/2 functions as a “double-blade razor” that first eliminates the acetyl groups from acetylated Lys residues and then removes the methyl group from Lys4. By contrast, the biochemical mechanisms underlying LSD1 involvement in transcriptional activation are not firmly established and remain an open problem for future research.
Our structural studies of the CoREST/LSD1 complex highlight a specific binding site for the histone H3 N-terminal tail and a catalytic machinery that is closely related to that of other flavin-dependent amine oxidases. These insights are critical for the development of demethylation inhibitors. A challenge for future studies will be to extend these structural investigations to visualize nucleosome binding by LSD1-containing protein complexes through biophysical methods and biocrystallography. Furthermore, the exploration of putative non-histone substrates and potential signaling roles of hydrogen peroxide produced by the demethylation reaction could lead to new paradigms in chromatin biology.

Supported by AIRC, MIUR-COFIN06, and Fondazione Cariplo

Signalling of HDAC inhibitors in cancer and non-cancer diseases

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Epigenetic deregulation of transcription, which contributes to diseases including cancer and cardiac hypertrophy, is reversible and the corresponding enzymes are promising drug targets. Indeed, histone deacetylase (HDAC) inhibitors are validated as anti-cancer drugs in advanced clinical studies. The availability of class II specific HDAC inhibitors allowed us to distinguish class I and class II-dependent effects. As little is known about the physiological functions of the human 18-member-HDAC-family, we initiated a pharmacological dissection of the roles of HDAC class II and class I and their inhibition in differentiation cellular models and in cancer. Intrigued by the anticancer action of class I HDAC inhibitors results obtained in cell lines, *in vitro* and *ex vivo* will be discussed which identify a '*complex*' disregulation at the level of the TRAIL promoter as responsible for its silencing in leukemias and modulation in cancer and establish a set of molecular markers which may predict epidrugs responsiveness in leukemias. Moreover insights on the effects of class II HDAC inhibition and its role on differentiation processes in myogenesis and adipogenesis will be also discussed.

Financial support: *EPITRON,* EU contract 518417; *APO-SYS*, EU contract 200767; *CANCERDIP*, EU contract 200620; Ministero Italiano dell' Università e della Ricerca PRIN 2006052835; Regione Campania, L. 5 annualità 2005.

Molecular genetics of Rett syndrome: when epigenetic signals go unrecognized

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Rett syndrome (RTT) is an X-linked progressive neurodevelopmental disorder causing mental retardation mainly in girls. Affected patients are characterized by a period of normal development followed by a rapid regression phase that leaves them with a profound mental handicap. Mutations in the *MECP2* gene, coding for a transcriptional repressor involved in chromatin remodeling, are the primary cause of Rett syndrome but are also found in patients affected by learning disability, neonatal encephalopathy, autism and mental retardation therefore making RTT paradigmatic for the study of autism spectrum disorders.
Recent experiments indicate that MeCP2-deficient neurons are not permanently damaged, since *Mecp2* reactivation leads to robust abrogation of advanced neurological defects in both young and adult animals. Although these results do not provide immediate therapeutic strategies for RTT, they establish the principle of reversibility of RTT and related disorders suggesting the necessity to develop therapeutical approaches.
In this communication we will summarize the fundamental discoveries of the last 9 years relevant to MeCP2 related neurological disorder and we will discuss the new challenges inspired by these discoveries. In particular, we will underline the importance of the identification of the critical factors that function downstream of MeCP2 as well as of the modifier genes that subdue the disease symptoms.
As Huda Zhogbi wrote "RTT story started in the clinic, but today has inspired many exciting basic science studies in neurobiology and epigenetics. It is anticipated that the next chapter in this story will involve translating some of the discoveries back to the clinic to benefit patients with RTT and patients with related neurological disorders".

Mechanisms in transcriptional control

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The regulation of gene expression is essential for the development and for driving the complex repertoire of different cell types and tissues in multicellular organism. This process must be finely controlled during the whole life of the organism and its deregulation results in a plethora of abnormalities, among them uncontrolled cell proliferation and cancer.
The knowledge of this process has changed enormously over the years. In addition to transcription factors, able to recognize specific cis-elements within the promoter, it is now clear that transcriptional control involves recruitment of co-activators and co-repressors to modulate the chromatin architecture. Moreover chromatin remodeling complexes acting in concert with histone modifying enzymes further change the nucleosome dynamics allowing recruitment of additional activators, Mediator complex, general transcription factors (GTF) and RNA polymerase.
In addition to protein-protein interactions a complex repertoire of posttranslational modifications are involved in regulating the ordered assembly and disassembly of these complexes and therefore transcription start and elongation. In this context an emerging role is played by the phosphorylation dependent prolyl-isomerization driven Pin1. This protein specifically recognizes and catalyzes the cis/trans inter-conversion of Ser-Pro or Thr-Pro motifs when Ser or Thr are phosphorylated by proline directed kinases. Pin1 binding produces on the client protein a conformational change that impacts on its function. Many key targets of Pin1 are transcription factors involved in cell cycle control, apoptosis, immune mediators but also elements of the transcription machinery such as the carboxy terminal domain (CTD) of RNA polII. Some Pin1 targets upon prolyl-isomerization are protected from degradation (e.g. p53, p73 etc.) other substrates instead are more efficiently degraded. The role of this protein in transcriptional control and in particular in modulating the functions of the tumor p53 will be discussed.

Sustained oscillations of functional NF-κB encode optimal gene expression programs

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In mammalian cells, various signals such as hormones, cytokines, and cell-cell interfaces elicit changes in gene expression mediated by inducible transcription factors. Many latent transcription factors, like the inflammatory NF-kB family, also induce expression of genes that provide feedback loops upon their signalling pathways. The negative feedback genes are generally thought to functionally terminate the signalling action of the transcription factor. These feedback loops also create the potential for the transcription factor activity to oscillate from the cytoplasm to the nucleus and backward over hours. However, oscillations have never been described in detail in mammalian living cells and the functional significance of such oscillations is unknown. Are they a mere side effect of the pathway settling back to the basal state, or are periodic cycles in fact required for proper gene expression programs?

We monitored in vivo NF-kB activities using an NF-kB/p65-GFP knock-in system. Oscillations of p65 were sustained in most cells, with several cycles of transient nuclear translocation after TNF-α stimulation with a period of about two hours.

During these 'signaling cycles', the ability of NF-kB to scan and interact with the genome *in vivo* remained functional, from early to late cycles. Inhibition of negative feedbacks abolished the oscillations and genome-scanning activities.

We also investigated oscillations of NF-κB activity to determine if such complex dynamic patterns encode specific cellular signaling information. We found that expression of NF-kB target genes is profoundly altered when properties of the NF-kB dynamics are manipulated.

We can conclude that the 'classical negative feedback' is a major driver of this oscillatory dynamics, and such feedback genes do not simply function to terminate signaling. Instead, they may render transcription factors 'kinetically wise", enabling them to sense the kinetic specificity of differential signalling contexts and to orchestrate an optimal response that is exquisitely linked to the incoming signal.

Novel RNA polymerase III promoters and terminators in the human genome

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The role of RNA polymerase (Pol) III in eukaryotic transcription is commonly thought of as being restricted to a small set of highly expressed, housekeeping non-protein-coding (nc) RNA genes. Recent studies, however, suggests a higher-than-expected number and functional diversity of Pol III-transcribed genes. Starting from general knowledge of Pol III promoter and terminator sequences, we are investigating on the ways such elements are exploited in humans to generate a variety of ncRNAs with potential regulatory roles. We found evidence for a widespread utilization of both gene-external and gene-internal promoter elements, and for the existence of many non-canonical Pol III termination signals in the human genome.

Poised transcription factories prime silent uPA genes prior to activation

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The position of genes in the interphase nucleus and their association with functional landmarks correlate with active and silent states of expression. Gene activation is thought to require *de novo* association with active transcription factories, and can induce chromatin looping from chromosome territories. By means of high resolution chromatin immunoprecipitation (MN-ChIP) and immunocryo-FISH we have identified factories containing RNA polymerase II phosphorylated on Ser5, but not Ser2, a conformation associated with rapid transcriptional activation of inducible genes. We call these sites 'poised transcription factories'. We find that the urokinase-type plasminogen activator (uPA) gene exhibits a poised state in uninduced conditions in HepG2 cells and undergoes dramatic nuclear repositioning upon activation. We demonstrate that repositioning of the uPA gene from its chromosome territory has no influence on its association with poised or active transcription factories, showing that the exterior and interior of territories are equally competent for the association of genes with the transcription machinery. We identify two distinct types of transcription factory, poised and active, characterized by the presence of different forms of RNAP. The association of inducible genes with poised transcription factories is likely to contribute to the rapid and robust activation in response to stimuli.

A systems biology approach to study transcription factors in mammalian cells: an application to the TF p63.

Diego Di Bernardo

Telethon Institute of Genetics and Medicine & Dept Computer Science & Systems of the University of Naples "Federico II"

Genome-wide identification of bona fide targets of transcription factors in mammalian cells is still a challenge. We present a novel integrated computational and experimental approach to identify direct targets of a transcription factor. This consists in measuring time-course (dynamic) gene expression profiles upon perturbation of the transcription factor under study, and in applying a novel “reverse-engineering” algorithm (TSNI) to rank genes according to their probability of being direct targets. Using primary keratinocytes as a model system, we identified novel transcriptional target genes of TRP63, a crucial regulator of skin development. TSNI-predicted TRP63 target genes were validated by Trp63 knockdown and by ChIP-chip to identify TRP63-bound regions in vivo. The integrated experimental and computational approach described we developed is readily applicable to other transcription factors in mammalian systems.

The anti-inflammatory profile of the histone deacetylase inhibitor ITF 2357

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The hydroxamic acid derivative ITF 2357 is an inhibitor of histone deacetylases (HDAC). Similarly to other HDAC inhibitors it has a cytotoxic and proapoptotic activity on tumor cells *in vitro* as well as *in vivo*.
ITF 2357, at doses lower than that are cytotoxic, has been demonstrated to have a potent inhibitory activity of the synthesis of pro-inflammatory cytokines by human white blood cells.
We determined its inhibitory activity against purified recombinant human HDAC Class I and II isoforms and the results obtained indicate that ITF 2357 is a pan-inhibitor of HDAC similarly to the well-known inhibitor SAHA, although its potency appear to be greater than that of SAHA.
In vitro experiments allowed us to conclude that the compound inhibits the Th1 response and increases the production of anti-inflammatory cytokines produced by Th2 lymphocytes.
Pharmacokinetic studies indicate that ITF 2357 has a good oral availability with a half-life of several hours, prompting us to evaluate its efficacy *in vivo*.
The anti-inflammatory profile of the compound was confirmed in several animal models that mimic Th1-driven human pathologies. In particular, inflammatory bowel disease models and arthritis models were adopted and the results obtained indicate that ITF 2357 dose-dependently inhibits tissue damage increasing the Th2 response.
ITF 2357, in addition to its anti-inflammatory properties, is cytotoxic for different tumor cell lines similarly to SAHA and others HDAC inhibitors.
On this base we evaluated the potential application of ITF 2357 in tumor models where pro-inflammatory cytokines, in particular IL-6 and of IFNγ, play a role such as in the interaction of multiple myeloma cells with stromal cells and in inflammation-mediated bowel tumors.
ITF 2357 inhibits the production of cytokines and of the angiogenic factor VEGF from mesenchymal cells co-cultured with myeloma cells, suggesting its clinical development in multiple myeloma patients. Moreover, in a mouse model of bowel inflammation ITF 2357 significantly reduced tumourigenesis.
The overall results indicate that the anti-inflammatory and proapoptotic activity of ITF 2357 should be a promising approach for tumor treatment.

Role of PI3-kinase signaling in steroid action

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Sex-steroid hormones, like growth factors, stimulate in breast cancer cells a complex signaling network through a rapid and direct association of steroid receptors with Src, the p85-regulatory subunit of PI3-K and other signaling effectors. Activation of these pathways is a key for the interplay between steroid-triggered signaling and the cell cycle machinery. Such an activation triggers cyclin D1 transcription, p27 nuclear exclusion and S-phase entry of target cells.
The critical role of estradiol-activated signal transduction is corroborated by recent findings showing that PI3-K/Akt/FKHR pathway modulates the estradiol-dependent nuclear export of estradiol receptor (ER) which is associated with FKHR. Blocking of this process impairs the S-phase entry in hormone stimulated breast cancer cells.

In non reproductive cells, androgen stimulates PI3-K pathways and activates small GTP-binding proteins and cell migration. The antiandrogen Casodex and inhibitors of Src and PI3-K prevent these hormonal effects. Remarkably, androgen stimulation does not induce receptor nuclear translocation and receptor-dependent transcriptional activity in NIH 3T3 cells, although they express the classical murine androgen receptor. The very low amount of androgen receptor present in NIH 3T3 cells is apparently responsible for the absence of receptor-dependent gene transcription.

The increasing knowledge of non genomic action of steroid receptors is providing new insights into steroid action and therapeutical approaches to mammary and prostate cancers.

Signal-dependent control of epigenetic modifications during skeletal myogenesis

Puri P.L

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Cell commitment to specific lineages and terminal differentiation entail an extensive nuclear reprogramming, leading to the selective expression of specific subsets of genes. The epigenetic modifications underlying this reprogramming are directed by extrinsic signals, such as developmental cues, during embryogenesis, and paracrine factors released in the regeneration microenvironment, during adult life. We have investigated the mechanism by which these signals are integrated at the chromatin level and interpreted by the components of the transcription machinery, to coordinate activation and repression of gene transcription during skeletal myogenesis. Our studies revealed that extracellular-signal activated p38 pathway coordinates the epigenetic modifications that establish the chromatin conformation repressive and permissive for the transcription of distinct subsets of genes, by directing the chromatin redistribution of the SWI/SNF chromatin remodeling complex and the Polycomb group, during myoblast differentiation into myotubes. These studies also revealed a novel mechanism of SWI/SNF recruitment to the chromatin of muscle loci. We show that BAF60c, a structural component of the SWI/SNF complex, forms a chromatin-bound complex with MyoD to prime muscle genes for transcription; p38-dependent phosphorylation of BAF60c is the signal by which the SWI/SNF complex is recruited to muscle genes, incorporates BAF60c and activates transcription. This evidence reveals an unanticipated two-step regulation of SWI/SNF recruitment to the chromatin and assigns to BAF60 subunits a new role as chromatin sensors of chromatin signaling. Finally, we show that pharmacological manipulation of the p38 signaling to distinct chromatin targets can be exploited to promote muscle regeneration *in vivo*.

Selected Oral Presentations

Session 'The RNA revolution'

An autoregulatory loop mediated by miR-21 and PDCD4 controls the AP-1 activity in RAS transformation

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The transcription factor AP-1 plays key roles in tumorigenesis, by regulating a variety of protein-coding genes, implicated in multiple hallmarks of cancer. Among non-coding genes, no AP- 1 target has been described yet. MicroRNAs (miRNAs) are negative post-transcriptional regulators of protein coding genes. miRNA expression signatures are highly relevant in cancer and several tumor-associated miRNAs (oncomirs) play critical roles in oncogenesis. Here we show that the antiapoptotic miRNA miR-21, which represents the most frequently upregulated oncomir in solid tumors, is induced by AP-1 in response to RAS. By analyzing validated miR-21 targets, we have found that the tumor suppressors PTEN and PDCD4 are downregulated by RAS in an AP-1- and miR-21- dependent fashion. We further show that, given the role of PDCD4 as negative regulator of AP-1, the miR-21-mediated downregulation of PDCD4 is essential for the maximal induction of AP-1 activity in response to RAS. Our data reveal a novel mechanism of positive autoregulation of the AP-1 complex in RAS transformation and disclose the function of oncomirs as critical targets and regulators of AP-1 in tumorigenesis.

MicroRNAs 17-5p/20a/106a control monocytopoiesis through AML1 targeting and M-CSF receptor upmodulation

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MicroRNAs (miRNAs or miRs) are conserved ~22 nucleotide non-coding RNAs: they repress protein expression at post-transcriptional level, mainly by annealing with the 3′ UTR of the target mRNA, thus interfering with its translation and/or stability. MiRNAs play important roles in the regulation of basic cell functions, including proliferation, differentiation and apoptosis. Importantly, oncogenesis has been linked to deregulated expression of miRNAs, which act as tumor suppressors or oncomirs and may contribute to tumor invasion.
We investigated the role of microRNA (miR) 17-5p, 20a and 106a in monocytic (Mo) differentiation-maturation. In unilineage Mo culture generated by hematopoietic progenitor cells these miRs are downmodulated, whereas the transcription factor AML1 is upmodulated at protein but not mRNA level. Since miR-17-5p/20a/106a bind the AML1 mRNA 3′UTR, their decline may unblock AML1 translation. Accordingly, miR-17-5p/20a/106a transfection suppresses AML1 protein expression, leading to M-CSF receptor (M-CSFR) downmodulation, enhanced blast proliferation and inhibition of Mo differentiation-maturation. Treatment with anti-miR-17-5p/20a/106a causes opposite effects. Knockdown of AML1 or M-CSFR by siRNA mimics the action of miR-17-5p/20a/106a, confirming that miR-17-5p/20a/106a targets AML1, which promotes M-CSFR transcription. In addition, AML1 binds the miR-17-5p-92 and -106a-92 cluster promoters and transcriptionally inhibits miR-17-5p/20a/106a expression. These studies indicate that monocytopoiesis is controlled by a circuitry involving sequentially miR-17-5p/20a/106a, AML1 and M-CSFR, whereby miR-17-5p/20a/106a function as a master gene complex interlinked with AML1 in a mutual negative feedback loop.

AN RNA POTENTIALLY REGULATING 4q35 GENE EXPRESSION IN FSHD

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Facioscapulohumeral dystrophy (FSHD), the third most common myopathy, is an autosomal dominant disorder whose genetic locus maps on the sub-telomeric region of chromosome 4 (4q35). FSHD is not due to a mutation in a protein-coding gene, but is rather caused by the reduction in the copy number of a repeated sequence (D4Z4) that leads to the epigenetic up-regulation of 4q35 genes FRG2, FRG1 and ANT1. Notably, mice over-expressing FRG1 develop a pathology strikingly similar to FSHD. Thus, it is important to define the molecular pathway responsible for 4q35 genes up-regulation in FSHD.
Healthy individuals carry 11-100 repeats whereas FSHD patients show less than 11 D4Z4 units. Paradoxically, the complete loss of the repeats has no pathological phenotype, strongly suggesting that at least one repeat is required for FSHD development.
We investigated for transcripts deriving from D4Z4. We found that D4Z4 generates a transcript selectively in FSHD muscles. Interestingly, even if this transcript contains an ORF we could not detect the protein in our system, moreover the RNA is strictly associated to the chromatin fraction and its expression correlates with 4q35 genes up-regulation. Most importantly, a specific knock down of this transcript results in the down-regulation of 4q35 genes, indicating a functional role of this RNA.
Experiments are on the way to characterize the transcript and the molecular mechanism by which it can regulate 4q35 gene expression in FSHD.

A new class of non-coding RNAs associated with 3′ untranslated regions of mRNAs

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The importance of non-coding RNAs (ncRNAs) in controlling gene expression is becoming increasingly evident. However, except for some well characterized examples, such as miRNAs, Xist and Air, the function of most non-coding transcripts is still to be determined. Moreover, while small regulatory RNAs can be relatively easily classified on the basis of their length, secondary structure, and biochemical pathway, the classification of long "mRNA-like" ncRNAs has been problematic.

Here we identify a large class of non-coding transcripts that originate within the 3′UTR of at least one third of all genes in the mouse genome. We have several lines of evidence from genome-wide bioinformatic analyses (EST coverage, CAGE data, chromatin state maps of active promoters) and from in-vitro studies (strand-specific RT-PCR, 5′RACE, Northern blot) showing that these 3′UTR-associated ncRNAs (uaRNAs) can be either linked or transcribed separately to the upstream protein-coding sequences. In addition, expression profiles obtained by custom-designed microarrays on three different developmental systems (myoblast differentiation, male gonadal ridge formation, embryonic stem cell differentiation) showed that uaRNA expression is highly regulated and tissue-specific, and might be either concordant or discordant with respect to the upstream coding region depending on the cell type and on the developmental stage. This observation is confirmed by in-situ hybridization experiments, which evidenced that uaRNA and the associated coding transcript might have different subcellular locations.

Our results highlight a further level of complexity at 3′UTRs, suggesting the presence of new regulatory mechanisms that control gene expression during embryonic development. Our data have also important implications for the design of in-situ hybridization and microarray probes as well as for the interpretation of gene expression data

Session 'The Epigenome and Gene Regulation'

Genome-wide survey of chromatin accessibility and dynamics during hematopoietic stem and progenitors differentiation

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A major goal in genomics is to understand how genes are regulated in different tissues, stages of development and diseases(1). The key feature distinguishing epigenomic from genomic information is the dynamic pattern of epigenetic features, which impose cell-type specific expression, regulating cell function(2). Genome accessibility in the context of cellular chromatin is a well-established epigenetic feature of active regulatory DNA(3). In this study, we have used primary human CD34+ cells to reveal changes in accessibility during myeloid differentiation. To overcome the limitations of DNaseI-based approach, we have developed a novel large-scale approach using restriction enzymes (RE) to probe and sequence accessible DNA (454 high throughput sequencig was used). Among these sequences, we identified known and novel cis-regulatory elements (validated by ChIP and reporter assays), including enhancers, silencers, insulators and promoters. Genome-wide analyses indicated that low nucleosome density is a common feature of gene promoters, even when poorly transcribed (as inferred by gene expression profile). A significant fraction of such elements (60%) were located at intergenic sites and repeated DNA, suggesting an active role in gene regulation. Finally, we mapped large genomic domains (0.5-1,2Mb) with differential accessibility during early step of hematopoiesis, a largely unexploited way to read chromatin accessibility.

The autoimmune regulator PHD finger binds to non-methylated histone H3K4 to activate gene expression.

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Methylation of lysine residues on histone H3 N-terminal tails is a critical factor in transcriptional regulation. Recently, the plant homeodomain (PHD) finger domain has emerged as a module that transduce histone-lysine methylation events, such as in BPTF and ING2 where it binds preferentially histone H3 trimethylated at lysine 4 (H3K4me3), or in BHC80, where it specifically recognizes histone H3 unmodified at lysine 4 (H3K4me0).
The AutoImmune REgulator (AIRE) protein is expressed in thymic medullary epithelial cells, where it promotes the expression of tissue-restricted antigens, and mutations in its gene cause autoimmune polyendocrinopathy candidiasis ectodermal dystrophy. AIRE contains two PHD fingers,PHD1 and PHD2, and the solution structure of AIRE-PHD1 was previously determined by our laboratory. By biochemical and biophysical studies, we demonstrated that AIRE interacts with histone H3 through its first plant homeodomain PHD finger and preferentially binds to non-methylated H3K4 (H3K4me0).The greater binding affinity of AIRE–PHD1 for H3K4me0 was determined by both isothermal titration calorimetry and tryptophan fluorescence spectroscopy and isothermal titration calorimetry, yielding dissociation constants of ~4M. This interaction is specifically abrogated by methylation of H3K4. Heteronuclear NMR spectroscopy and site directed mutagenesis were used to map the binding determinants to AIRE-PHD1.
Interestingly AIRE-PHD2 does not interact with histone H3 neither in the methylated nor in the unmodified form. We are currently determining the solution structure of AIRE-PHD2 and by a proteomic approach we are searching for possible AIRE-PHD2 interactors. Finally we investigated the functional relevance of AIRE-histone interaction showing that, in vivo, AIRE binds to and activates promoters containing low levels of H3K4me3 in human embryonic kidney 293 cells. This provides a new link between histone modifications and regulation of tissue-restricted antigen expression in thymus.

NF-Y, a transcriptional regulator with H2A-H2B structural and functional properties.

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Transcription is governed by DNA-binding proteins that recognize specific sequences in regulatory regions, in the context of chromatin. Histone tails have a plethora of different post-translational modifications which can influence the establishment of other epigenetic marks. Associated with the establishment of active chromatin, H2B K120 mono-ubiquitination is required for H3K4me3 and H3K79me2 methylations.
One of the most widespread promoter elements is the CCAAT box, bound by the NF-Y trimer. Two of NF-Y subunits have an H2A-H2B-like structure. By using a NF-Y DNA-binding dominant negative we established the causal relationship between NF-Y binding and positioning of methyl marks: the decrease of NF-Y binding in CCAAT promoters leads to a decrease of H3K4me3, H3K79me2, a removal of components of the H3K4 methylating MLL complex and a transcriptional repression. The H2B-like subunit of NF-Y is mono-ubiquitinated *in vivo* in Lysine 140 of the histone fold domain, which structurally corresponds to the H2B K120. Micrococcal nuclease Re-ChIP assays allowed the separation of NF-Y-Ub, in promoters, from H2B-Ub and its E3 ubiquitin-ligase, hBRE1A, in transcribed areas. A NF-YBK140R mutant causes an impaired deposition of H3K4m3 and H3K79m2 in CCAAT box promoters and transcriptional repression of NF-Y targets genes, acting as a dominant negative.
We propose a scenario whereby NF-Y is a “variant” sequence-specific histone a-la-H2B that marks transcription units containing the CCAAT recognition sequence, making chromatin locally accessible to the histone modifying machines that would account for further modifications.

Session 'Mechanisms in Transcriptional Control'

GFP-engineered embryonic stem cell lines as a tool for the identification of key regulators of cardiac and neural differentiation.

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Embryonic stem (ES) cells provide the basis for establishing an in vitro model for early mammalian development, facilitating the identification and characterization of genes and factors implicated in the modulation of different differentiation processes. Given the complexity of the biological system, one approach to identify regulators of ES cell differentiation is the exploitation of high throughput screening technologies. However, such powerful technologies are strictly dependent on the development of sensitive and reproducible cell-based assays for screening. In this perspective, we have generated a set of engineered ES cell lines stably expressing the Green Fluorescent Protein (GFP) under the transcriptional control of well-characterized tissue-specific promoters: a) the MLC-2v promoter, a DNA regulatory region upstream of the Myosin Light Chain gene which confers heart specific expression; b) the promoter region of the gene encoding the Ta1-Tubulin product, which is transcriptionally active in developing neurons. The GFP-expressing cell lines obtained have been characterized in detail by the means of immunolocalization of specific markers and FACS analysis. Our results show that: the GFP accumulates exclusively in differentiating ES cells, namely cardiomyocites in the case of MLC-2v cell line, and neural cells (developing neurons) in the case of Ta1 cell line; the GFP fluorescent signal can be quantified in live cells with specific and reproducible kinetics. Moreover, we have also assessed that these GFP-expressing ES cell lines can be used in biological assays specifically developed for high throughput screening procedures, with an easily detectable read-out in live cells. Thus, the described ES cell lines represent a powerful tool that will be exploited for large scale screenings of collections of bioactive molecules; in particular, we will use different libraries of small interfering RNAs for down-regulating gene expression.

PIM1-dependent phosphorylation of histone H3 at serine 10 is required for MYC-dependent transcriptional activation and oncogenic transformation

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The serine/threonine kinase human Pim1 (hereafter PIM1) cooperates with human c-Myc (hereafter MYC) in cell cycle progression and tumorigenesis. However, the nature of this cooperation is still unknown. We show that, after stimulation with growth factor, PIM1 forms a complex with the dimer of MYC with MAX (Myc-associated factor X) via the MYC BoxII (MBII) domain. MYC recruits PIM1 to the E boxes of the MYC-target genes FOSL1 (FRA-1) and ID2, and PIM1 phosphorylates serine 10 of histone H3 (H3S10) on the nucleosome at the MYC-binding sites, contributing to their transcriptional activation. MYC and PIM1 colocalize at sites of active transcription, and expression profile analysis revealed that PIM1 contributes to the regulation of 20% of the MYC-regulated genes. Moreover, PIM1-dependent H3S10 phosphorylation contributes to MYC transforming capacity. These results establish a new function for PIM1 as a MYC cofactor that phosphorylates the chromatin at MYC-target loci and suggest that nucleosome phosphorylation, at E boxes, contributes to MYC-dependent transcriptional activation and cellular transformation.

Clustering of transcription factor binding sites: Understanding the rationale behind a recurring genomic theme

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Clustering of transcription factor binding sites (TFBS) at cis-regulatory regions is a widespread feature of higher eukaryotic genomes. However, the rules underlying the usage in vivo of homotypic clusters of TFBS are still ill-determined. To address this issue we use as a model NF-kB, a family of TFs regulating the expression of genes implicated in the inflammatory and immune response. Large-scale bioinformatics analysis has demonstrated that NF-kB binding sites (kB sites) occur in homotypic clusters in the cis-regulatory regions of NF-kB-dependent genes. However, due to the close proximity of kB sites within clusters, which makes impossible to resolve single-kB site occupancy by chromatin immunoprecipitation, the in vivo correlation between site occupancy and transcriptional activity of a gene cannot be investigated by standard biochemical means. We thus devised an alternative approach. We designed a physical model of a NF-kB-dependent promoter containing a cluster of kB sites, which relies on the equilibrium statistical mechanics of the one-dimensional Ising model and is strongly grounded on experimental evidence regarding NF-kB nuclear and binding dynamics. Once fed with an a priori hypotheses concerning the usage of kB sites in the clusters, it returns an experimentally accessible output (transcription level of the gene vs. NF-kB concentration, as a function of the number of kB sites in the promoter region), which can be used to test the hypothesis. We present here the preliminary experimental tests of the model, concerning the measurement of gene transcription as a function of NF-kB concentration for a panel of endogenous NF-kB target genes bearing different numbers of kB sites, and the generation of cell lines with a single-copy integration in the same chromosomal location of a panel of in-vitro generated promoter mutants bearing different numbers of NF-kB binding sites driving the expression of GFP.

Session 'Transcription, Differentiation and Disease'

IDENTIFICATION AND CHARACTERIZATION OF CRIPTO ANTAGONISTS TO IMPROVE THE USE OF STEM CELL-BASED THERAPY IN NEURODEGENERATIVE DISORDERS

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Recent breakthroughs in stem cell research underscore the importance of controlling stem cell differentiation for the success of cell-based transplantation therapies in neurodegenerative diseases, such as Parkinson Disease (PD). Embryonic Stem (ES) cells serve as a source for deriving the optimal dopaminergic (DA) cells to restore the nigrostriatal system; however, our knowledge of the molecular mechanisms inducing neural fate in ES cells is still incomplete and thus their differentiation is poorly controlled. The EGF-CFC protein Cripto is a GPI-anchored multifunctional protein and a key player in the Alk4/Nodal/Smad2 signaling pathway that control ES cells fate. Disruption of Cripto in ES cells results in increased dopaminergic differentiation and reducted tumor formation, upon transplantation in rat models of PD. We have exploited a novel experimental approach based on the use of combinatorial chemistry combined to ES cell differentiation. By using an ELISA-based assay, we previously identified a tetrameric tripeptide able to block efficiently Cripto/Alk4receptor interaction. Here we show that this blocking peptides, by inhibiting the endogenous Cripto signaling, impairs cardiac differentiation and redirects the differentiation of ES cells to a neural fate. In addition, the Cripto/Alk4 blocking peptide promotes ES cell differentiation to dopaminegic neurons and reduce teratoma formation.
All together our data provide evidence that, through the addition of small, non-toxic activators/inhibitors of signaling pathways, the differentiation of pluripotent ES cells might be controlled for the production of specific cell types, suitable for the use in animal model of human disease. Worth noting, the ability to direct ES cell fate solely through the use of extracellular factors, without the need to manipulate ES cells genetically, may permit a direct extension of this strategy to human cells.

Regulation of Dlx5 and Dlx6 gene expression by p63 is involved in the EEC and SHFM congenital limb defects

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The congenital malformation Split -Hand-/Foot Malformationt Malformation (SHFM, or ectrodactyly) is characterized by a medial cleft of hands and feet and missing central fingers. Five genetically distinct forms are known in human; the most common (type-I), is linked to deletions of DSS1 and the distalless-related homeogenes DLX5 and DLX6. As Dlx5;Dlx6 double knockout mice show a SHFM-like phenotype, the human orthologs are believed to be the disease genes. SHFM-IV and Ectrodactlyly-Ectodermal dysplasia-Cleft lip (EEC) are caused by mutations in p63, an ectoderm-specific p53-related transcription factor. The similarity in the limb phenotype of different forms of SHFM may underlie the existence of a regulatory cascade involving the disease genes. Here we show that p63 and Dlx proteins colocalize in the nuclei of the Apical Ectodermal Ridge (AER). In homozygous p63Brdm2 (null) and p63EEC (R279H) mutant limbs the AER fails to stratify and the expression of four Dlx genes is strongly reduced; interestingly the p63+/EEC and p63+/- hindlimbs, which develop normally and have a normally stratified AER, show reduced Dlx gene expression. The p63+/EEC mutation combined with an incomplete loss of Dlx5-Dlx6 alleles leads to severe limb phenotypes, not observed in mice with either mutation alone. In vitro DNp63a induces transcription from the Dlx5 and Dlx6 promoters, an activity abolished by EEC and SHFM-IV mutations, but not by AEC mutations. ChIP analysis shows that p63 is directly associated to the Dlx5 and Dlx6 promoters. Thus, our data strongly implicate p63 and Dlx5;Dlx6 in a pathway relevant in the aetio-pathogenesis of SHFM.

Polycomb complex shapes the higher order of D4Z4 chromatin structure during differentiation of normal and FSHD muscle stem cells

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscolar disorder. FSHD involves a complex cascade of epigenetic events following contraction of a D4Z4 repeat located on chromosome 4q35.2 (FSHD locus). Previous work has indicated that transgenic mice overexpressing FRG1, a gene proximal to the deletion, showed a phenotype resembling the FSHD disease. However, increased expression of FRG1 in FSHD patients has not been a uniform finding and up to now, several studies have failed in identifying the molecular mechanism affecting the FSHD locus functionality.
We took advantage of ChIP/MeDIP and 3D immuno-FISH assays as complementary approaches to depict the higher order of chromatin organization of 4q35.2 region during myogenic differentiation of healthy and FSHD myoblast and mesoangioblast stem cells. We found that FRG1 undergoes to muscle specific regulation through a two-step activation mechanism, whereby removal of H3-K27 methylation and Polycomb complex components precedes MyoD recruitment on the FRG1 promoter; intriguingly, the same chromatin structure and PcG recruitment were contemporaneously found on D4Z4 array, rendering the Polycomb complex the first molecular player that link s FSHD locus to myogenic differentiation. Moreover, D4Z4 H3-mK27 signals were strongly reduced in FSHD myoblasts in respect to controls, suggesting the severe impairment of the PcG complex recruitment. Nevertheless, molecular alterations of the D4Z4 array do not have in FSHD myoblasts an effect in cis on FRG1 gene expression. These observations evidence a role of 4q35 D4Z4 in muscle differentiation, probably through inter-chromosomal interactions.

POSTER PRESENTATIONS

EXPRESSION OF THE ALPHA SEVEN NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNIT DUPLICATE FORM IS DOWNREGULATED IN THE MONOCYTIC CELL LINE THP-1 ON TREATMENT WITH LIPOPOLYSACCARIDE

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The 7 nicotinic acetylcholine receptor subunit is partially duplicated (7dup) in the genome and here we investigate its expression in three monocyte like cell lines; THP-1, U937 and Mono-Mac-6. Despite qualitative PCR revealing the presence of the classic 7 gene in these cell lines, we found using Real Time PCR, exclusive expression of the 7dup subunit which was downregulated in THP-1 on treatment with LPS. This reduction in gene expression was seen to be mediated by a direct transcriptional mechanism reliant on NF-B, as pharmacological block experiments using its specific inhibitor parthenolide, prevented the same observed reduction in 7dup transcript.

RETINOIC ACID CONTROLS PHOX2A EXPRESSION BY MEANS OF A DUAL REGULATORY MECHANISM

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The specification of neuronal identity is the result of the interaction between two distinct classes of determining factors: extrinsic factors, including secreted or cell membrane-associated signals in the local environment, and intrinsic factors that often consist of transcription factors cascades. PHOX2A is a homeodomain protein that participates in the network regulating the development of autonomic ganglia. We used an undifferentiated human neuroblastoma cell line to show that retinoic acid, a well-established extrinsic factor that profoundly affects the differentiation of sympathetic neurons at different developmental stages, regulates PHOX2A expression by means of a dual effect: it starts by acting as a positive regulator of gene expression, and later triggers a process, completely evident after 48 hours of treatment, culminating in the selective proteasome-mediated degradation of the PHOX2A protein, whereas the corresponding mRNA remained up-regulated. The persistence of PHOX2A protein, induced by treatment with proteasome inhibitors, resulted in a selective dis-regulation of the trascription of the Dopamine--hydroxylase, a well characterized PHOX2A target gene. This suggests that the expression of PHOX2A must be finely regulated during development in order to direct neurons towards the terminal noradrenergic differentiation.

CELL SPECIFIC INTEGRATION PROFILE WITH NO IN VIVO SKEWING IN MATURE T CELLS FOLLOWING PBL GENE THERAPY FOR ADA-SCID

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Non random integrations of gammaretroviral vector is usually thought to be related with a promoted clonal fitness in vivo. Other works link these preferences to specific host conditions in vitro at the time of vector integrations. To address these issues we analyzed the host-vector interaction in peripheral blood T cells derived from retrovirally transduced mature T cells. The results were compared with the integration profile of T cells differentiated in vivo from CD34+ hematopoietic stem/progenitor cells transduced with the same MLV vector. We mapped vector integrations in four ADA-SCID patients who received infusions of peripheral blood T cells transduced with a MLV vector encoding ADA. In vitro transduced T cells (n=88) and ex vivo derived peripheral blood T cells 3 to 11 years post-infusion (n=101) were analyzed by LAM-PCR to identify genome-vector junction. Our results confirmed the classical non-random distribution of MLV-derived retroviral insertion sites (RIS), with a preference for transcription start sites and gene dense regions. We next studied the functional profile of genes hit by or immediately upstream/downstream from a RIS. The analysis of expression levels of hit genes at the time of transduction showed an in vivo preference for RIS landed in regions transcriptionally active in T cells (61% vs 49). In addition both pre- and post-transplant RIS displayed a striking tendency for vector integrations landing in/near genes involved in specific pathways of T-cell function without any particular in-vivo bias. In contrast, the functional profile of RIS-associated genes was significantly different in T cells derived from in vivo differentiation of transduced stem/progenitor cells, specifically lacking the over-representation for genes related to T-cell functions and activation. These results suggest that retroviral integration profiling is cell-type specific and the ex-vivo RIS distribution seems to mirror directly this preference without particular biases.

Histone demethylation in stem cell commitment

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The methylation of histone tails on lysine residues is one of the most significant mechanisms of gene regulation. In particular patterns of methylation at lysine 4 and 27 of histone H3 determine states of gene activation and repression that are developmentally regulated and are thought to underlie the establishment and maintenance of lineage specific gene expression programs. The recent identification of several histone demethylases (LSD1 and members of the JmjC-domain protein family) that are specific for certain lysine residues has suggested a potential mechanism to account for the dynamic regulation of histone methylation patterns during development. The observation that lineage specific genes that are repressed in embryonic stem cells (ESc) by Polycomb-mediated H3K27 methylation are derepressed during lineage commitment hinted at the role of regulated H3K27 demethylation during development. Furthermore several loci in ESc including key development regulators are characterized by the simultaneous presence of H3K4 and H3K27 trimethylation, a configuration described as bivalent domain. In undifferentiated cells this unusual combination of opposing marks is thought to keep genes expressed at low levels but poised for later activation. At the end of the transition from ESc to neural stem cells (NSc) a large fraction of these bivalent domains appears to have been resolved leaving monovalent signatures of either activation (H3K4) or repression (H3K27).
Starting from a comprehensive expression profiling of JmjC-domain proteins during the differentiation of ESc into NSc we report that Jmjd3, recently identified by us and others as a H3K27 specific demethylase, is required for commitment to the neural lineage. Through a combination of in vitro and in vivo approaches we show that Jmjd3 directly controls key regulators and markers of neurogenesis and highlight different modalities through which the dynamics of H3K27 trimethylation is related to transcriptional output during lineage specification.

Identification of genes involved in osteoblast differentiation with an shRNA-based approach

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Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate in a variety of cell types, such as osteoblasts, adipocytes, and chondrocytes; moreover, they can differentiate also in myocytes and neurons. Nonetheless, the molecular mechanisms that regulate these differentiation processes are not completely understood.
At this aim, MSCs and MSCs-derived cell lines constitute a valuable tool for the analysis of the basic mechanisms underlying differentiation of mesenchymal tissues, such as bone, cartilage and fat. Furthermore, study of differentiation mechanisms in these tissues can lead to relevant results for applications in the therapy of several pathologies, such as discontinuous bone fractures, ligament ruptures and several skeletal disorders.
We have devised rapid and reliable protocols for differentiation of MSCs in adipocytes, chondrocytes and osteoblasts. We plan to focus our activities towards osteoblast differentiation; at this aim, we are silencing specific mRNAs using an shRNA library, composed of at least two silencing constructs for each transcript, in a 96-well-plates-based screening strategy. We are focusing our study on a mouse shRNA library and an in-vitro system. With this methodology we plan to identify genes that are possible candidates to have a role in normal tissue differentiation.
We have identified to date a set of putative candidates that include genes with a previously identified role in osteoblast differentiation and a number of genes whose role in this process is still unknown. We plan to confirm the involvement of the above-mentioned genes in vitro and in vivo and evaluate potential applications to bone-related pathologies.

Nitric oxide-dependent activation of a target promoter by the transcription factor DNR from Pseudomonas aeruginosa

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The response of cells to gases like oxygen, carbon monoxide (CO) and nitric oxide (NO) is a key biological process which involves different proteins ranging from transcription factors to enzymes. The interaction of gases with transcription factors often leads to a wide reprogramming of the cell metabolism (both in prokaryotes and eukaryotes). The molecular details of the activation process is still largely unknown (1).
In Pseudomonas aeruginosa the Dissimilative Nitrate respiration Regulator (DNR) controls the expression of the nitrite- and NO-reductase genes under low oxygen tension and in the presence of NO (2). Thus, this protein is a key regulator of the NO-dependent anaerobic metabolism, which is essential for Pseudomonas aeruginosa pathogenesis in cystic fibrosis patients.
DNR belongs to the CRP-FNR superfamily of transcription factors which respond to different signal molecules, such as cAMP, redox balance and gases like CO and NO.
To identify the molecular bases of the interaction of DNR to the target promoters in response to NO, we have expressed in E. coli a recombinant form of the protein. Two putative target promoters (from E.coli and P. aeruginosa), both containing a consensus FNR-box, have been tested by beta-gal assays; moreover, a truncated DNR mutant without DNA-binding domain has also been tested. The assay was performed both aerobically and anaerobically in the presence of N-oxides. Since spectroscopic evidence suggest that DNR can bind heme in vitro (3), we are also investigating the NO-dependent activation of target promoters by DNR mutants and in a E.coli strain defective in heme biosynthesis.

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The absence of HMGB1 decreases the total amount of core histones and changes the overall chromatin compaction

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High Mobility Group Box-1 (HMGB1) is a highly abundant and evolutionarily conserved non-histone chromatin component with the ability to bind and bend DNA. The protein is involved in fundamental nuclear processes including nucleosome sliding, transcription, replication, V(D)J recombination, and DNA transposition. Recent work has shown that mammalian HMGB1 and its related yeast proteins, Nhp6A/B, play important roles in maintaining genome stability both in mammalian cells and in yeast.
Here we show that nuclei from Hmgb1-/- MEFs have a wild-type/whole complement of genomic DNA but contain only 80% of the nuclear amount of core histones (H2A, H2B, H3), variants (H2AX) and H1 as compared to wt MEFs (by immunoblotting and SILAC quantification). We found the same result with Nhp6a/b yeast mutants. Hmgb1-/- cells are larger (1.5x beta-actin content) and the nuclei are bigger (1.5x, P=0.0001) suggesting a less compacted chromatin structure. By micrococcal nuclease assay, the accessibility of chromatin in Hmgb1-/- nuclei appears altered, with regions of either increased or reduced sensitivity to nuclease. We calculated that approximately 20% less DNA is protected by nucleosomes after nuclease digestion. The size of the DNA protected by the nucleosome is very slightly increased in the nuclease-resistant fraction. Accessibility results have been confirmed in HeLa cells where HMGB1 protein was stably knocked-down by siRNA.
To our knowledge, this is the first observed instance where the global content of nucleosomes in the genome is significantly altered. The altered chromatin packaging in Hmgb1-/- MEFs may account for the high incidence of mitotic defects (46%) and the increase in DNA single and double strand breaks (Comet Assay) after gamma-ray irradiation and genotoxic treatments. Nonetheless, these cells are indeed viable with only 80% of the normal content of histones.

Histone methylation and demethylation in lineage establishment and maintenance.

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The epigenetic regulation of the genome is central to the function of multicellular organisms. To understand how the lineages of adult animals are maintained to ensure tissue homeostasis we have to characterize the epigenetic programs that underlie the balance between stem cell renewal and differentiation. The methylation and demethylation of histones on lysine tails is one of the most significant mechanisms of epigenetic regulation through which single genes as well as entire chromosomal domains are activated or silenced.
Our aim is to investigate the function of selected HMTs and histone demethylases (HDMs) in the establishment and maintenance of cell identity and their deregulation in cancer, using the mouse as a model system. Among about 40 known HMTs in man and mouse, we decided to take as a point of our interest the Mll family members: Mll – the first described methyltransferase - and Mll2 which lead to transcriptional activation through the methylation of H3K4.
In order to dissect the in vivo function of Mll2, I am using conditional Mll2 knock-out mice generated in the group of Francis Stewart according to the 'knock-out-first' strategy. This strategy allows knocking out a gene not only ubiquitously but also in time-and-space specific manner using inducible or tissue specific Cre recombinase.
In order to investigate the role of Mll and Mll2-dependent H3K4 methylation in cell lineages, I have chosen one of the best known systems of cellular differentiation: the maturation of B cells, a system well-suited to uncover the function of Mll2-dependent H3K4 methylation in a well-defined and relevant cell lineage. Within a collaboration with Stefano Casola I have access to a panel of stage specific Cre-expressing mouse lines which are my tools to ablate gene function at defined stages of B-cell maturation.

Identification and characterization of new Cripto downstream targets during ES cell differentiation

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In vitro differentiation of Embryonic Stem (ES) cells proceeds as a developmental continuum and this could be considered as a powerful model system to identify key regulators of mammalian development. Cripto, the founder member of the EGF-CFC family, is expressed both in the Embryonic Stem (ES) cells and during the early phases of embryo development while, in the adult, it is reactivated in a wide range of epithelial cancers. The involvement of Cripto in physiological events associated with development and pathological events linked to tumorigenesis, points out for the search of genes regulated by Cripto. We are currently studying the expression profile of cripto-/- ES cells stimulated with recombinant Cripto protein for 24 hrs, by using microarray technology. The time window selected reflects early steps in the differentiation process that ultimately yield high numbers of beating cardiomyocytes. By using this experimental approach we have identified new cripto-responsive genes involved in the early phases of stem cell differentiation and we are currently studying the functional interaction of Cripto with the most promising genes: the G protein coupled receptor, apj and its ligand, apelin. Our data indicate that apj and apelin act as previously unforeseen downstream targets of Cripto signaling in cardiomyogenesis, thus raising a new role for apj and apelin in cardiac ES cell differentiation.

Cross-regulation of PHOX2A and PHOX2B transcription factors in the development of the Autonomic Nervous System

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PHOX2A and PHOX2B are homeodomain transcription factors important for the development of the entire Autonomic Nervous System (sympathetic, parasympathetic and enteric divisions).
PHOX2B is absolutely required at the early stages of development of autonomic ganglia: its expression is triggered by bone morphogenic proteins (BMPs) secreted by the dorsal aorta; it acts as a repressor of negative signals induced by Notch, a BMPs antagonist, and supports MASH1 with which it coordinates the expression of downstream factors as PHOX2A and dHAND, a process necessary for the regulatation of the expression of c-RET, TH (tymidine hydroxylase) and DBH (dopamine hydroxylase) genes which are characteristic of the cathecolaminergic phenotype.
During the specification of neuronal identity, control of temporal and spatial expression of PHOX2A and PHOX2B is fundamental, and many studies over the last few years have tried to elucidate the exact molecular mechanisms involved in regulation of their expression. We have demonstrated previously that PHOX2B regulates the transcription of the PHOX2A gene by directly binding and transactivating its promoter. We also characterised the PHOX2B promoter and demonstrated by means of biochemical and functional assays that most of its transcriptional activity is sustained and maintained by an auto-regulatory mechanisms in which PHOX2B binds and transactivates its own promoter.
Chromatin immunoprecipitation (ChIP) assays showed that PHOX2A also participates in the transcriptional complex assembled on the PHOX2B promoter. The functional significance of this is under further investigation and the results of the various approaches used are the topic of the present poster.

Culture conditions select different mesenchymal progenitors from adult mouse bone marrow

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Development of murine models of several pathologies increases the interest in the isolation and characterization of mouse bone marrow stromal cells (BMSCs) for preclinical studies. In the present study, we provide a characterization of different cell populations derived from murine bone marrow; we have isolated and expanded cells in four conditions adding the following components: a) bone fragments conditioned medium; b) bone fragments conditioned medium with EGF and PDGF-AA; c) MS-5 conditioned medium and d) NIH3T3 conditioned medium. We observed that mouse BMSCs can be found in low abundance. The progenitors obtained with the different culture conditions showed an immunophenotypic profile consistent with the absence of hematopoietic markers; in addition, they were positive to the Sca I and CD44 antigens. Little differences were observed among the different progenitors for the expression of CD117, CD13 and H2-k b.
In vitro differentiation showed tripotential differentiation abilities only when the MS-5, or bone fragments conditioned medium was added to the cells; the progenitors selected in the condition b showed loss of adipogenic differentiation ability, cells treated with NIH3T3 conditioned medium (condition d), in contrast, showed a high adipogenic potential. Our results indicate that different isolation and culture conditions in the first passages allow the isolation of different progenitors from murine bone marrow. Presence of different cytokines, such as leukemia inhibitor factor (LIF) that we found to be expressed in NIH3T3, is probably responsible of the different differentiation abilities that we observed. Complete characterization of cytokine and factors needed for lineage specification will be relevant for application of BMSCs in clinical settings.

Post-transcriptional regulation of skeletal muscle differentiation by microRNAs.

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Primary myoblasts expressing a ts mutant of the Src tyrosine kinase differentiate in a temperature-dependent manner. Activation of ts-Src in post-mitotic myotubes induces a dramatic reduction in the accumulation of muscle-specific mRNAs and a profound disorganization of muscle fibers. The stability and the translational efficiency of mRNAs often depend on short sequences present in the 3'UTRs and recognized by specific microRNAs. To identify novel microRNAs involved in muscle-specific mRNA regulation we have performed microRNA expression profiling in Src-expressing myoblasts and myotubes. We found that miR-221 and miR-222 are strongly down-regulated as differentiation proceeds and their expression increases again following Src reactivation in post-mitotic myotubes. p27kip1 cdk inhibitor is known to be up-regulated during myogenic differentiation and is a validated target of both miR-221 and miR-222 in mammalian cells. We confirmed that p27kip1 is a target of miR-221 and miR-222 also in primary avian myoblasts and found that inhibition of its accumulation by ectopic expression of miR-221 and miR-222 in differentiating myoblasts induced a delayed exit from the cell cycle and a reduced accumulation of muscle-specific proteins in myotubes. The role of miR-221 and miR-222 in post-transcriptional regulation of p27kip1 and in myogenic differentiation will be discussed.

Decitabine, differently by DNMT1 silencing, exert its antiproliferative activity through p21 upregulation in Malignant Pleural Mesothelioma (MPM) cells.

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Malignant pleural mesothelioma (MPM) is a locally aggressive neoplasm principally linked to asbestos fibres exposure. There are strong evidence that associate this ambiental pollutant with induction of DNA breaks, aberrant chromosomes segregation and important chromosomal rearrangements, considered crucial events in malignant transformation. A considerable contribution to cellular transformation in MPM is also given by the presence of high genomic instability, as well as by the increased DNA methylation, and consequent decreased expression, of tumor suppressor genes.
In this study it was first demonstrated the decreased methylation level of pericentromeric DNA sequences and a paradoxical concomitant increased expression of DNMT1, the most expressed DNA methyltransferases in MPM cells, DNMT3a and all five isoforms of DNMT3b. Thus, we compared two experimental strategies, DNMT1 silencing and usage of a demethylating agent (5-aza-2′-deoxycytidine or Decitabine), both theoretically able to revert the locally hypermethylated phenotype and considered potential future therapeutic approaches for MPM. Interestingly, both strategies substantially decrease cell survival of MPM cells but Decitabine, differently from DNMT1 silencing, exerts its antiproliferative activity inducing a p53-independent p21 upregulation, associated with cell cycle arrest at the G2/M phase. These results indicate that the two approaches act probably through different mechanisms and, thus, that DNMT1 silencing can be considered an effective alternative approach to Decitabine for cancer treatment.

Study of the expression of the β1C Integrin in prostate cancer

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The β1C integrin is an alternatively spliced variant of the β1 integrin subfamily that at variance with its wild-type counterpart, *i.e.* the β1A integrin, inhibits cell proliferation in prostate cancer cells. We have recently shown that transcriptional, translational and post-translational processes contribute to the selective loss of β1C integrin during prostate malignant transformation. Here, we investigated whether neoadjuvant hormonal therapy may affect β1C mRNA expression in prostate cancer.
Normal prostates were obtained from patients who underwent radical cystoprostatectomy for bladder cancer. Neoplastic prostates were obtained from patients who underwent radical prostatectomy; and received neoadjuvant therapy or not. The β1C mRNA level was measured by Northern hybridization experiments and the β1C integrin gene transcriptional activity was measured by nuclear Run-on.
In this way we have shown for the first time an increase of β1C mRNA expression in patients who underwent a 1-month neoadjuvant therapy in comparison to the patients without any therapy. On the contrary, there were not significant variations in the level of β1C integrin expression measured in the patients after 3- and 6-months of neoadjuvant hormonal therapy.
Furthermore, in the patients who underwent a 1-month therapy we have shown an increase of the gene transcriptional activity possibly responsible for the up-regulation of the β1C integrin mRNA levels.
In conclusion our data seems to show that short-time administration of androgen-deprivation therapy interferes with β1C integrin expression, thus indicating that androgen-mediated mechanisms act through β1C involving pathways.
The exact characterization of the mechanism/s that regulate the expression of this adhesion factor in the prostate cancer cells will gain further insight about the molecular mechanisms involved in the tumor progression and about possible molecular targets for the development of new therapeutic strategies in prostate cancer.

Analysis of the transcription machinery of the microRNA genes

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It is becoming clear that non-coding RNAs play an important role in the regulation of gene expression. They are defined as ss-non coding RNAs 22nt in length, processed from longer precursors mainly transcribed by the RNA polymerase II (pol II).
More than 400 human mature microRNAs have been identified so far by computational approaches (http://microrna.sanger.ac.uk), but little is known about the structure and regulation of their transcriptional units (TU).
miRNAs are commonly associated with complex transcriptional loci and they are often transcribed as part of the coding or non-coding host genes, taking advantage of their transcriptional regulation.
On the contrary, intergenic microRNA genes are believed to be transcribed independently. Unfortunatly, we have limited understanding about the regulation of this new class of genes : in particular their promoter regions and TU have so far been poorly characterized.
To understand the mechanism of miRNA biosynthesis, we undertook the characterization of the transcriptional machinery of independently transcribed microRNAs and compared it with a canonical pol II apparatus.
We performed ChIP assay to examine whether the RNA Polymerase II, TFIID (TBP and TAFs) and TFIIB physically interact in vivo with the promoter region of miRNAs constitutively espressed in HeLa cells.
In parallel, we also analysed microRNAs specifically up-regulated during granulopoiesis and neurogenesis.
Our data indicate that, althought the basal transcription machinery is recruited on the microRNA host gene promoters, intergenic microRNA genes are not transcribed by a canonical Pre-Initiation-Complex (PIC), suggesting that evolution has diversified this complex to perform important specialized functions.

Coordination mechanisms in the synthesis of ribosomal components.

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Ribosomes include a large number of components (80 ribosomal proteins and 4 RNA molecules in eukaryotes) assembled mostly in the nucleolus with final steps in the cytoplasm. This process, which requires more than 170 accessory factors, is generally coordinated to cell growth and it is sensitive to several cellular stresses.
Alterations of ribosome structure or function are involved in several diseases. For instance, mutations in ribosomal protein (RP)S19 gene have been identified in about 25% of patients of Diamond-Blackfan Anemia (DBA), a congenital hypoplastic anemia associated to various physical malformations. Although there is evidence of coordination in the production of RPs and rRNAs, the molecular mechanisms underlying such regulation have not been clarified. The aim of this work is to study the effect of the decrease of an RP on the synthesis of other ribosomal components. As an experimental system, we are using K562 cells inducible for small interfering RNA (siRNA) against RPS19 (developed by S. Karlsson to study DBA mechanism).
In a first series of experiments we have analysed the steady state level of ribosomal subunits and of different RPs after induction of RPS19 interference. We observed that the down-regulation of RPS19 caused a dramatic reduction in amounts of small subunits and mature 80S ribosomes and an excess of large subunits. Moreover, western blot analysis showed that the ratio between RPS19 and the other RPs of the small subunit is constant. At the same time the ratio between RPS19 and RP of the large subunit decreases, consistent with the observed unbalance between the ribosomal subunits. Experimental and published data suggest that the equilibration in the level of RP of the small subunit occurs at the post-translational level. We are now investigating if there are regulations at different levels (transcriptional, post-transcriptional, translational) in response to RPS19 deficiency.

Molecular characterization of CDKL5, a novel kinase involved in Rett Syndrome and infantile spasms

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Rett Syndrome (RTT) is an X-linked neurological disorder and represents the second cause of mental retardation in females. Mutations in the methyl-CpG binding protein (MeCP2) gene cause the majority of RTT cases. Recently, mutations in the cyclin-dependent kinase-like 5 (CDKL5) gene have been found in some RTT patients with the Hanefeld variant. Pathogenic mutations in CDKL5 were also found in females with early signs of developmental delay and epileptic seizure onset, further reinforcing the importance of this gene in mental retardation and epilepsy.
We are characterizing the role of CDKL5 in the nervous system thereby clarifying the molecular mechanisms involved in disease onset. We have previously shown that CDKL5 and MeCP2 function in a common pathway; in fact, they associate and the kinase is able to mediate the phosphorylation of MeCP2. This suggests that CDKL5 might also play an indirect role in RTT acting as a modifier gene that, by regulating MeCP2 functions, is able to influence disease severity in patients with mutations in MeCP2.
Here we will show that both CDKL5 expression and its subcellular localization are highly modulated during embryogenesis and post-natal development. In addition, in adult mouse, CDKL5 protein level and its cytoplasmic/nuclear fraction are tightly regulated in the different brain areas. Moreover, we will present data demonstrating that CDKL5 shuttles between the nucleus and the cytoplasm and that an active nuclear export mechanism is involved in regulating its localization. Our analysis suggests that the C-terminal tail of the kinase is responsible for the cytoplasmic localization. Importantly, we will show that a number of RTT truncating mutations, found in this region, are mislocalized in the nucleus. We believe that this analysis will contribute in drawing a phenotype-genotype correlation in patients with mutations in CDKL5 and in understanding the role of CDKL5 as an MeCP2 modifier gene.

NUCLEO-CYTOPLASMIC TRANSPORT FACTORS REGULATE MITOSIS AND INTERPHASE RE-ENTRY

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Regulated transport of RNAs and proteins across the nuclear envelope is a fundamental process that underlies the gene expression programme of every cell. This process is regulated by the GTPase Ran and its effectors that act as transport vectors: importins (for proteins that have a nuclear function) and exportins (for RNAs and proteins that are active in the cytoplasm). Importin beta is the major vector of nuclear protein import in interphase. Growing evidence indicate that Ran and importin beta play pleiotropic functions: in addition to regulating nucleocytoplasmic transport, they have roles in mitotic spindle assembly and in nuclear envelope organization in reconstitution systems.
In this work we investigate the mechanisms through which Ran and importin beta operate in mitosis in human cells in vivo. We have found that full-length importin beta causes structural mitotic abnormalities when overexpressed in human cells; time-lapse imaging also reveals dynamics defects, with prolonged prometaphase duration and unstable chromosome alignment at the cell equator. These defects trigger the mitotic checkpoint response. A deletion mutant that retains only a central portion of importin beta exacerbates these defects and is detrimental for progression through all mitotic stages: in addition to hindering chromosome alignment, this mutant also dramatically delays metaphase and impairs mitotic exit in cells that manage to progress past metaphase. Thus, importin beta regulates several steps of mitotic division and exit through distinct functional domains that interact with specific targets. These results begin to pinpoint novel unsuspected functions of transport factors in control of chromosome segregation and genome stability.

Structural characterisation of the transcription factors Prep1, Pbx1 and HoxB1 in complex with DNA.

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Homeodomain proteins constitute a large family of eukaryotic DNA-binding proteins that regulate transcription of a broad range of developmentally important genes. These proteins share a 60 amino acid DNA-binding domain that is conserved in sequence, structure and mechanism of DNA-binding. While monomeric homeodomain proteins exhibit a limited affinity and ability to discriminate between different DNA sequences, their specificity is significantly enhanced through the cooperative binding to DNA with other DNA binding partners. Prep1, which belongs to the TALE (three amino acids loop extension) family of homeodomain proteins, forms a complex with Pbx1 independent of DNA binding. Prep1 and Pbx1 form trimeric complexes with HoxB1, which is known to play an important role in development. Through structural characterisation of the homeodomain proteins Prep1, Pbx1a and HoxB1 and their interaction with DNA we hope to reach an understanding of the structural basis underlying the functional DNA target specificity. In a broader perspective, the determination of these structures will be valuable in the characterisation and understanding of the functions of this class of transcription factors during processes of development and cancer.

EGFR expression identifies distinct populations of glioblastoma multiforme cancer stem cells with different tumorigenic potential.

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The most accepted theory of tumorigenesis states that only a subset of cells, i.e. the cancer stem cells (CSCs), is able to sustain tumor formation. However, different research groups provided evidence that in murine leukemias and lymphomas different populations of tumor-initiating CSCs exist and represent the majority of tumor cells.
Here we demonstrate that different subpopulations of CSCs can be identified on the basis of EGFR expression and in vitro mitogen independence in glioblastoma multiforme (GBM) CSC lines and in GBM tumor specimens. These subpopulations of CSC are characterized by long-term self-renewal, multipotency and tumor initiating capacity. When isolated either from GBM specimens or from established CSC lines based on different EGFR expression, CSCs expressing the highest level of EGFR form tumors faster than those characterized by low or negative levels of the same protein. Interestingly, these same CSC populations are different for the expression of ErbB family receptors and activation of their pathways, suggesting that GBMs are made of distinct CSCs, each of which defined by peculiar functional and molecular characteristics.
By removing mitogens from culture medium, growth factor-independent (GF-I) CSCs could be isolated from GF-D CSCs as well as directly from GBM specimens. All these GF-I CSCs display an extraordinary increase in tumorigenic potential as compared to their growth factor-dependent (GF-D) counterpart. Notably, in some of these lines, the GF-I phenotype correlates with the up-regulation of EGFR.
Thus, the availability of different patient-derived GBM CSCs will give us the possibility to perform gene expression analysis by micro-array technology. By this approach, we might be able to identify genes involved in the regulation of GBM CSC biology. In fact, only through good knowledge of the molecular pathways that specifically sustain tumor growth and formation, we may plan appropriate therapeutic strategies to eradicate the GBM.

Fat cadherin and its involvement in retinal degeneration.

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Fat is a cell adhesion molecule belonging to the cadherin family and conserved across species. It is considered to be a tumor suppressor that plays a role in cell proliferation and organ size since lethal mutations display hyperplastic overgrowth of cells during development. How Fat regulates these mechanisms is not fully established but it has been shown recently to act through the Hippo tumor suppressor pathway. One of the first molecules shown to interact with Fat is Atrophin, the unique Drosophila homolog of human Atrophins. The protein is linked to a Human Neurodegenerative Disease, DRPLA, caused by a poly-Q expansion within the Atrophin-1 gene. Fat and Atrophin control similar processes during development of neuronal tissues and display strong genetic interactions. Our analysis shows that Fat mutant clones, also in the fly eye, display age dependent abnormalities in the morphology of the photoreceptors, the neuronal cells that constitute retinal tissue. The severity of the phenotype increases with the age. These defects suggest a role of Fat in neuronal homeostasis and are enriched when removing one copy of Atrophin in the same background for Fat. Since Fat is involved in regulating the Hippo tumor suppressor pathway, we also analyzed the phenotype of the retina in fly eyes mutants for some other activators of this pathway. Preliminary data suggest a possible link between the Hippo pathway and retinal degeneration. In conclusion we argue that Fat, alone or in cooperation with Atrophin, is involved in retinal neurodegeneration and that it could act to prevent retinal neurons death also with the help of other players.

ANALYSIS OF MBP PROMOTER AND ITS TRANSCRIPTIONAL FACTORS TO POTENTIATE REMYELINATION

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The differentiation of oligodendrocytes (OL) and the activation of myelin gene expression may regulate the amount of myelin produced during the repair of a demyelinated lesion. Myelin Basic Protein (MBP) is one of the most important myelin proteins in CNS because it is required for normal development and myelination. It is expressed only by OL and its dosage is primarily transcriptionally regulated and axonally modulated. A binding site between –124 and –118 nt is able to bind a protein complex, MEBA, (Myelinating glia-enriched DNA binding activity) only from OL nuclear extracts. MEBA appears and increases as oligodendrocytes differentiate. We identified Pur-alpha and Pur-beta transcription factors as two of the components of MEBA. Functional and binding analysis of mutations throughout the MEBA binding site showed that alterations of MEBA binding reduced promoter activation by as much as 70% in OL. Pur proteins appear to be necessary but not sufficient for MBP expression, as their overexpression in cultered OL, either undifferentiated or differentiated, does not markedly increase promoter activation even when Pur-alpha and Pur-beta are fused to the VP16 activation domain. However, immunolocalization analyses suggest that the fusion proteins remain in the cytoplasm. We hypothesize that the weak activation could be due to lack of nuclear transport, maybe from another factor of MEBA. A candidate could be Sox10. We have identified a hypothetical binding site for Sox10 next to MEBA site, and it is known Sox10 is able to bind Pur-alpha in another cell-type. Moreover, Sox10 is required for OL terminal differentiation. Thus, MEBA contains Pur-alpha and -beta, and may cooperate with other proteins, such as Sox10, in order to activate the MBP promoter as oligodendrocytes terminally differentiate.

Which alpha integrin pairs with beta1 to form the Schwann cell receptor required for axonal sorting?

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During peripheral nervous system development Schwann cells segregate large caliber axons destined to be myelinated during the process of "axonal sorting". Axonal sorting is necessary for subsequent myelination, and requires signals deriving from both axons and the extracellular matrix. Genetic alterations in mice have shown that laminins and beta1 integrin are required for axonal sorting. Thus a receptor containing beta1 integrin links laminin to the Schwann cell cytoskeleton to enable them to ensheathe axons. To form laminin receptors, beta1 integrin can pair with different alpha integrin chains, many of which are expressed by Schwann cells. The goal of this study is to identify the partner of beta1 integrin in the receptor involved in axonal sorting.
Alpha6 integrin is highly expressed in Schwann cells during axonal sorting and alpha6beta1 integrin is a laminin receptor. Thus, we ablated alpha6 integrin specifically in Schwann cells using the Cre/LoxP system in mice. Alpha6 conditional null mice are viable and fertile. Despite complete recombination of the alpha6 gene in sciatic nerves, and absence of the alpha6 protein in Schwann cells, sciatic nerves and spinal roots surprisingly do not present sorting abnormalities. To address possible redundancy/compensation we are analyzing the expression of other alpha integrins (alpha1, 2, 3, 5, and 7) and we will produce appropriate single or double-null mice. This study will elucidate the general mechanism of Schwann cell sorting of axons, a fundamental step for peripheral nervous system development and myelination

Dissection of the Myc-dependent transcriptional program in response to mitogenic stimuli.

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For many vertebrate cell types the G0S-phase progression is the major decision point regulating cell proliferation and execution of cell differentiation programs. This cell cycle progression can be induced in resting fibroblasts by restoration of mitogens.
Several immediate-early serum-responsive genes, such as c-myc, c-fos, or c-jun, encode transcription factors that are believed to drive induction of secondary mitogen-responsive genes. Consistent with this view, Myc function as a sequence-specific transcription factor that has been shown to be essential for this response, but the set of genes directly regulated and required in such conditions have remained obscure. The purpose of this study is to identify Myc-target genes playing critical roles in the G0S-phase progression. We took advantage of c-mycflox/flox 3T9 fibroblasts. In these cells, the c-myc open reading frame is flanked by loxP sites in order to obtain its deletion using an inducible Cre-ER recombinase activity. Using DNA microarrays, a gene expression profiling of serum response in murine fibroblasts either expressing or not expressing Myc was performed. We found a group of 93 genes induced by serum in a Myc-dependent manner. Seventy-four of these genes were directly bound by Myc during mitogenic response, as determined with a quantitative chromatin immunoprecipitation assay. Gene ontology analysis revealed that the group of genes was enriched for genes involved in metabolism of nucleotides and for those coding for ribosomal proteins and regulators of translation. This group of direct Myc-target genes likely constitutes the core transcriptional response built up by Myc during the G0S-phase progression. Next goal will be to understand if any of those genes is necessary for the cell cycle re-entry governed by Myc in response to serum. Accordingly we are now starting a functional screen, based on a lentiviral shRNA expression system, to knock-down each one of the identified Myc-target genes.

The role of histone H3 lysine-27 demethylase Jmjd3 in muscle cell differentiation

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Methylation of lysine 27 on histone H3 (H3K27me) by Polycomb Repressive Complex 2 (PRC2 complex) is associated with gene silencing in many cellular processes, such as development, differentiation and proliferation. The histone methylation was long considered to be irreversible process but recent identification of JmjC-domain proteins Utx and Jmjd3 as H3K27-specific demethylase showed that this modification is dynamically regulated enabling the activation of genes. Since PRC2 complex has been implicated in regulation of muscle gene expression (Caretti et al., 2004) we have decided to study the antagonistic function between methylation and demethylation of H3K27 during muscle cell differentiation. We are currently investigating the role of Jmjd3 in the activation of muscle genes and how signaling pathways regulate involvement of Jmjd3 during muscle differentiation. The details of this project as well as preliminary results will be presented.

Molecular and Functional Characterization of Tie2-expressing Monocytes (TEMs)

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We recently described a population of Tie2-expressing monocytes (TEMs), which circulate in mouse and human blood and specifically home to tumors, where they are required for angiogenesis and tumor growth. Yet, a detailed molecular analysis of TEMs is still lacking, and their lineage-relationship with other myeloid populations previously implicated in tumor growth remains to be clarified.
To this end, we compared the expression profile of tumor-derived TEMs with that of tumor-associated macrophages (TAMs) and related myeloid populations, using qPCR-based arrays interrogating ~300 genes in three different biological samples (n=3). Despite the global similarity between TEMs and TAMs, statistical analysis of the gene expression data revealed a several differentially expressed genes. Of note, many classical pro-inflammatory molecules, including IL12a, iNOS and TNFa were significantly down-regulated in TEMs. Conversely, many scavenger receptors (CD163, Stab1 and CD206) were up-regulated, together with LYVE1, SDF1, IGF1 and Neuropilin1, as compared to TAMs. These data strongly suggest that TEMs have unique features among TAMs.
We then investigated whether TEMs' anti-inflammatory phenotype could be reverted by exposing the cells to Th1 cytokine (IFNg+LPS). We found that TEMs were refractory to pro-inflammatory stimuli, whereas they retained the ability to respond to Th2 cytokine (IL4).
Interestingly, TEMs were not able to mount allogenic responses in mixed lymphocyte reaction (MLR) assays. In this regard, TEMs behaved similarly to myeloid-derived suppressor cells (MDSCs), a population of immune-suppressive cells that can be isolated from the spleen of tumor-bearing mice. These findings, together with the gene expression data, suggest that TEMs may also create an immune-privileged environment that promotes tumour growth. Future studies employing the appropriate mouse models will explore this intriguing scenario and possibly unravel new targets for anti-cancer therapies.

Novel lentiviral vectors efficiently deregulate oncogene expression by insertional mutagenesis - implications for high throughput oncogene tagging in solid tissues

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High throughput retroviral insertional mutagenesis led to the discovery of several novel oncogenes in mouse models of hematopoietic and breast cancer. Integrated retroviruses trigger oncogenesis by deregulating nearby oncogenes. Mapping their integration sites in tumor cells allows the identification of the virus-tagged cancer promoting genes. However, this tool for oncogene discovery is limited due to restricted tissue tropism.
Recently we have developed a lentiviral vector (LV) carrying strongly active retroviral enhancer/promoter sequences in the long terminal repeats (LTR). Mice transplanted with Cdkn2a-/- tumor prone hematopoietic stem cells treated with this LV develop lymphoma/leukemia significantly earlier than controls. Analysis of vector integrations in tumors shows that more than 35% of the vector integrations targeted known cancer genes, some of which were significantly overexpressed with respect to matched control tumors. These data indicate that the mechanism of LV oncogenesis recapitulates the one described for retroviruses.
We decided to exploit the wider tissue tropism of LVs to develop a new insertional mutagenesis platform for high-throughput oncogene screenings in the liver. We developed a modified LV containing hepatospecific enhancer/promoter sequences in the LTR. To increase the sensitivity to LV-mediated liver mutagenesis, we used double knockout Cdkn2a-/-, IfnaR1-/- mice. Cdkn2a deficiency enhances the sensitivity to oncogenic mutations, while IfnaR1 deficiency enhances the hepatocytes transduction in vivo. LV administration to newborn mice induced hepatocarcinomas (HCC) in 30% of animals, whereas none was found in untransduced controls. Vector integrations in HCC tissue targeted repeatedly Braf and other genes involved in hepatocyte growth and survival suggesting that a strong oncogenic selection occurred.
This new LV-based approach provides a novel mutagenic tool that may open new avenues to oncogene discovery in HCC and solid tumors.

MOLECULAR CHARACTERIZATION OF VASCULAR SMOOTH MUSCLE CELLS IN ZEBRAFISH EMBRYOS

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Vascular smooth muscle cells (vaSMC) are highly heterogeneous cell type with different embryonic origins, properties and gene expression profiles. Vascular SMC are subject of great interest because of its important role in normal and pathological vascular processes, like atherosclerosis, retinopathies, tumor angiogenesis. Understanding origins, differentiation and plasticity of smooth muscle cells, has been hampered by difficulties in observing these cells in vivo and in performing defined genetic and experimental manipulation of the smooth muscle cells in currently available model organisms. Here, we characterized vascular smooth muscle development in zebrafish using microscopic, molecular and genetic tools. We traced vascular smooth muscle in the ventral aorta (VA) and outflow tract, dorsal aortae (LDA), anterior mesenteric arteries (AMA) from lateral plate mesoderm during vascular development in zebrafish. We showed that zebrafish vascular smooth muscle cells share many of the morphological, molecular and functional characteristics of the smooth muscle cells found in higher vertebrates. These results indicate that the zebrafish system is an useful cellular and genetic model for studying vascular myogenesis in vertebrates.

Genome-Wide Discovery of STAT3 Functional Binding Sites

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STAT3 is a transcription factor playing a crucial role in inflammation, immunity and oncogenesis. Direct targets so far identified are in limited number, not sufficient to explain its crucial functions. To improve our understanding of STAT3 transcriptional network we developed a computational approach for the discovery of STAT3 functional binding sites. We generated a Positional Weight Matrix (PMW) from 54 functional validated STAT3 binding sites and used a log-likelihood ratio scoring function to identify potential binding sites of predicted affinity. Theoretical affinity was experimentally confirmed by EMSA competition assays.
To identify those sites more likely to be bound in vivo and thus to be functional, phylogenetic footprint was carried out between H.Sapiens and eight different vertebrate species using a sequence alignment-based method and a whole-genome comparison. The results obtained were crossed with a list of genes differentially expressed in MEFs plus or minus STAT3, obtained by microarray analysis. Predicted binding sites present in putative regulatory regions of differentially regulated genes were assigned a score based on the degree of species conservation and on predicted affinity.
In order to validate the method, the top ten STAT3 binding sites thus identified, plus other seven candidates selected on the basis of their biological function, were subjected to Chromatin Immunoprecipitation (ChIP) analysis using chromatin from wild type or STAT3-/- MEFs either untreated or treated with Oncostatin M to activate STAT3. A high percentage of sites was found to be bound by STAT3 in vivo (16 out of 18 tested) indicating a very low rate of false positive predictions. Moreover, expression analysis revealed a good correlation between STAT3 in vivo occupancy and levels of corresponding mRNAs. Therefore, our computational approach can provide a potent tool to identify novel STAT3 transcriptional targets.

DNA Methyl-Transferase inhibitors and Histone De-Acetylases inhibitors contrast leukemias reactivating the same apoptosis pathways.

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The connection between Epigenetic and Cancer is, nowadays, clear: aberrant DNA methylation and/or aberrant histone modifications are associated with a high number of different cancers. For instance, aberrant epigenetic modulations are constantly found in APL, as a consequence of PML/RAR altered recruitment of HDACs and DNMTs. For this reason Epigenetic Drugs are being investigated in both basic and clinic research. HDAC inhibitors and DNMTs inhibitors have been investigated as single drugs, while common features and potentials have been only superficially described. Our work sought to validate Epigenetic Drugs as a new family of anti-cancer agents, using leukemias as a model. We demonstrated that 5'aza 2'-deoxycytidine (DAC), a DNMT inhibitor in vivo prolongs survival in APL mice (as well as AML mice) by reactivating a silent Apoptosis Extrinsic Pathway through the re-expression of TRAIL/APO2L. It is known that the same gene is reactivated also by a HDAC inhibitor, VPA, in the same leukemia models, supporting the hypothesis that drugs targeting different epigenetic modulators can act on the same mechanisms. We also described a hypermethylated region of TRAIL promoter that can be suggested as the direct link between DAC epigenetic modulation and TRAIL re-expression, since CpGs are demethylated and histone H3 acetylation gets significantly increased in this region after drug in vitro treatment. Moreover both VPA and DAC positively cooperated with All Trans Retinoic Acid (ATRA) in contrasting leukemia after ATRA treatment suspension, suggesting that both HDACi and DNMTi can be candidate secondary drugs in differentiating therapies, again having comparable potentials even if acting on different epigenetic targets. We concluded that HDACs and DNMTs are viable new anti-cancer targets, and that drugs acting on them can be considered as one Family of Drugs, with similar properties and potentials.

p38 MAPK signaling regulates Polycomb (PcG) activity during muscle differentiation

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The Polycomb group (PcG) and trithorax group (TrxG) genes are part of widely conserved cell memory system that prevents changes in cell identity by maintaining transcription patterns, throughout development and in adulthood. The balanced action of PcG and trxG genes controls the expression of developmentally and cell cycle regulated genes. Polycomb proteins form repressive complexes (PRCs) that function in the initiation and maintenance of gene silencing by catalyzing histone H3 methylation (H3K27) at target loci. EZH2, a member of PRC2 complex, was shown to regulate muscle gene expression acting as a classical PcG repressor in muscle progenitor cells. To date, several aspects of PcG function in muscle differentiation are still unknown, such as the role of SUZ12 another member of PRC2 complex required for EZH2 histone methyltransferase activity, and cellular signals that remove PRC2 complex from muscle genes promoting muscle gene expression and cell differentiation.
We observed that Suz12 is recruited to the promoter of several muscle-specific genes. Notably, while some of these genes are up-regulated after depletion of SUZ12, in keeping with a repressive function of this PcG protein, others are down-regulated under the same experimental conditions. This suggests the role of SUZ12, in both transcriptional repression and activation. Moreover, we found that the differentiation-activated p38 pathway is involved in displacement of PRC2 complex from its target genes in muscle progenitor cells by regulating the PRC2 activity.

Adherens junctions control tight junctions: VE-cadherin up-regulates claudin-5 by inhibiting FoxO1 and –catenin

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Intercellular junctions mediate adhesion and communication between adjoining cells. Although formed by different molecules, tight junctions (TJs) and adherens junctions (AJs) are functionally and structurally linked but the signalling pathways behind this interaction are unknown. We describe here a novel and cell-specific mechanism of cross talk between these two types of structures. We show that endothelial VE-cadherin at AJs up-regulates the TJ adhesive protein claudin-5. This effect is mediated by the release of the inhibitory activity of forkhead box factor FoxO1 and Tcf-4/-catenin transcriptional repressor complex. VE-cadherin acts by inducing FoxO1 phosphorylation through AKT activation and by limiting -catenin nuclear translocation. These results offer a molecular basis for the link between AJ and TJ and explain why VE-cadherin inhibition may cause dramatic increase in permeability.

Kinetochore and centromere are novel target for Gcn5p in yeast

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Epigenetic regulators like HATs (Histone Acetyltraferase) beyond regulating chromatin remodeling and gene expression are involved in many other cellular processes.
We have shown a novel role for Gcn5p required for the progression of cell cycle and at the metaphase to anaphase transition. We demostred that Gcn5p exerts a direct role in the control of faithful chromosome segregation by physical interacting to the centromeric region of yeast chromosomes. The screening of a wide collection of double mutants of kinetochore components disrupted in GCN5 highlighted genetic interaction demostrating a direct role for Gcn5p in centromere-kinetochore function.
Results will be presented showing how important is the regulation exerted by gcn5p on kinetochore and on the anctivation of mitotic checkpoint.

Finally, in search of novel selective inhibitors for Gcn5p we are developing a yeast based drug screening in order to identify gene targets for selected novel compounds.

Identification of new p63 targets in keratinocytes by ChIp on chip

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p63 is a transcription factor required for the development and maintenance of ectodermal tissues, including skin, limb and, in general, multilayered epithelia. The identification of its target genes is fundamental for understanding the complex network of gene regulation governing the development of these epithelia. Our lab employed two strategies for this task: expression profiling of si RNA p63 depleted human keratinocytes (HaCat cells) (1) and ChIP on chIP analysis on two different platforms (CpG island and promoter arrays) with specific p63 antibodies in HaCat cells. We previously reported 186 high confidence p63 targets which were validated in different biological assays.(2). A re-analysis of the chip-chip data with less stringent criteria enlarged this list of putative targets to almost 1000 new locations. Independent chIP validations confirmed that around 60-70% of these new targets are bound by p63 in vivo and a subset of these genes are changing in expression during in vitro differentiation of primary human keratinocytes. Moreover, functional annotation of this extended list highlighted some unexpected GO terms enrichments such as heart development and nucleo-cytoplasmic transport. Detailed location analysis and functional studies are on going on these particular subclasses of genes to understand the physiological role of p63 in these processes.

1. Testoni et al. Cell Cycle.;5(23):2805-11 (2006)
2. Vigano et al. EMBO J.;25(21):5105-16 (2006)

Mutational Analysis of Mammalian SR Protein Kinase 2 (SRPK2)

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Tissue-specific alternative splicing profoundly affects animal physiology, development and disease, and this is nowhere more evident than in the nervous system. Alternative splicing is a versatile form of genetic control whereby a common precursor messenger RNA (pre-mRNA) is processed into multiple mRNA isoforms differing in their precise combination of exon sequences. In the nervous system, thousands of alternatively spliced mRNAs are translated into their protein counterparts where specific isoforms play roles in learning and memory, neuronal cell recognition, neurotransmission, ion channel function, and receptor specificity. The essential nature of this process is underscored by the finding that its misregulation is a common characteristic of human disease such as neurodegenerative disorders.
Our approach to gain insight into the regulation of neuron specific pre-mRNA splicing is to focus on the characterization of the kinase SRPK2. This protein phosphorylates serine/arginine-rich domain (RS-domain)-containing proteins and is expressed almost exclusively in the nervous system. SR proteins are a family of pre-mRNA splicing factors that play an essential role in constitutive and regulated splicing. The activity in pre-mRNA splicing and the subcellular localization of SR proteins are modulated by the level of phosphorylation of their RS domain.
In order to understand how SRPK2 intracellular localization and activity are regulated, we have constructed deletion mutants, as well as loss-of-function and gain-of-function mutants in Ser and Tyr residues that were shown to be phosphorylated. These mutants have been characterized by transient transfection in SH-SY5Y neuroblastoma cells and analyzed by immuno-fluorescence microscopy. Moreover, the mutants have been characterized under oxidative stress condition generated by pharmacological treatment of SH-SY5Ycells.

ALTERNATIVE SPLICING AS POSSIBLE CONTRIBUTOR TO LSD1 EPIGENETIC TUNEABILITY IN THE MAMMALIAN NERVOUS SYSTEM.

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Chromatin represents the substrate of epigenetic regulation underlying several biological processes, including DNA replication, transcription, lineage commitment and cell response to environmental stimuli. Recent advances suggest chromatin involvement in the mammalian nervous system and its active remodelling consequent to synaptic activity, finally leading to the acquisition and dynamic maintenance of neuronal phenotype.
Mechanisms such as DNA methylation, ATP-dependent remodelling and post-translational modifications on basic N-terminal histone tails all contribute to the histone code, altering chromatin accessibility to transcriptional machinery.
In this context acts the enzyme LSD1 - Lysine Specific Demethylase 1 - whose epigenetic activity relies on specific demethylation of mono and di-methylated H3K4 residues, through an amino oxidase reaction, resulting in repression of target genes, which is achieved through concomitant recruitment of LSD1 and its associated factors, such as members of the CoREST-corepressor complex.
Although several efforts have been focused on LSD1 enzymatic activity and its cognate effectors, neither its function not its own regulation have been fully elucidated.
According to experimental data, LSD1 transcript can encompass several splice isoforms, some of which display a remarkable neuro-specific pattern of expression, while apparently retaining comparable activities.
The arousal of such splice isoforms in the earliest murine embrional stages and their progression over subsequent stages of SNC development account for LSD1 role in neural commitment. Moreover, maintenance of LSD1 splice variants in post-mitotic, terminally differentiated neurons, before and after depolarizing stimuli suggests that alternative splicing could possibly contribute to LSD1 epigenetic tuneability in the nervous system.