EUROPEAN SCIENCE FOUNDATION

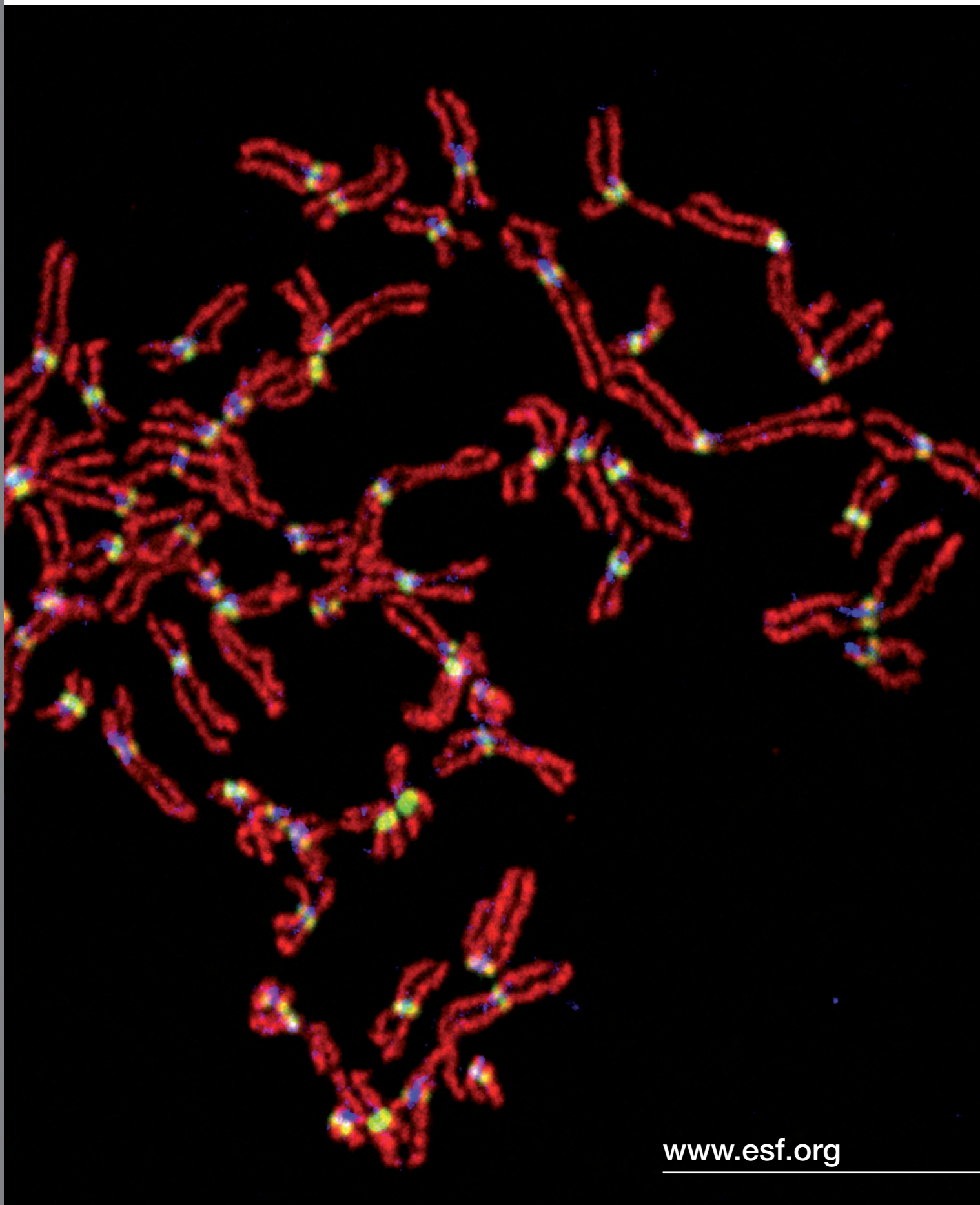
SETTING SCIENCE AGENDAS FOR EUROPE

EUROCORES Programme
European Collaborative Research

Conference EuroDYNA

DYNAMIC NUCLEAR ARCHITECTURE AND CHROMATIN FUNCTION

Mendel Center in Brno • Czech Republic **12-14 October 2006**



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One of the major challenges in biology is to understand how the genome orchestrates gene expression of the many thousand genes it encodes. To tackle this issue, the European Science Foundation (ESF) together with national funding agencies from eight European countries has set the stage for 41 research groups to join forces and to coordinate their efforts across Europe within the framework of the European Collaborative Research (EUROCORES) Programme EuroDYNA.

EuroDYNA aims at advancing our knowledge of the control of gene expression in nuclear organisation. To do this the Programme gathers and combines expertise in different fields such as dynamic chromatin structure and nuclear architecture, regulation of gene expression, RNA processing and transport as well as genome surveillance. Latest technologies in molecular biology and biochemistry are employed together with advanced microscopy, structural analysis and computational approaches in order to gain a deeper insight into how the nucleus operates.

There are nine Collaborative Research Projects (CRPs) under the umbrella of EuroDYNA which started their research in 2005. In addition to its multidisciplinary character, the Programme offers a wide range of networking opportunities to the entire EuroDYNA community; providing training possibilities and establishing a platform to stimulate new research initiatives between scientists with related yet slightly different scientific interests, and to promote collaboration with other national and European initiatives.

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Acknowledgement

This meeting, as part of the ESF EUROCORES Programme EuroDYNA, is supported by funds from the European Commission Sixth Framework Programme under contract no. ERAS-CT-2003-980409.

Cover picture:
Spread of human mitotic chromosomes stained for the proteins condensin (red), cohesin (blue) and the centromere specific histone, CENPA (green). Micrograph by Peter Lenart, IMP, Vienna.

Programme EuroDYNA Conference

Thursday 12 October

17:00 – 18:00 Mendel Lectures Series
(organized by Mendel Center)
John Gurdon
Wellcome/CRC Institute, University of Cambridge, UK
Nuclear reprogramming as a route to cell replacement

18:30 *Welcome drinks and buffet*

Session 1

Chair: **Pavel Kovarik**

19:20 Welcome and Introductory remarks
Pavel Kovarik and **Astrid Lunkes**

19:30 Plenary Lecture
Ulrich Laemmli
Depts. of Biochemistry and Molecular Biology, University of Geneva
Inside the nucleus

20:30 – 20:55 **Jiri Bartek**
Institute of Cancer Biology, University of Copenhagen
Dynamics of DNA damage response pathways in human live cell

20:55 – 21:20 **Anna Friedl**
Physics Department, Technical University Munich
Radiation-induced Chromatin Alterations: Application of Ion Microirradiation

Friday 13 October

Session 2

Chair: **Niels Galjart**

09:00 – 09:25 **Leonie Kamminga**
Hubrecht Laboratory, Utrecht
Piwi proteins and small RNAs in the zebrafish germline

09:25 – 09:50 **Torben Jensen**
Department of Molecular Biology, University of Aarhus
Severely compromised transcription due to a 5' splice site mutation

09:50 – 10:15 **Maria Carmo-Fonseca**
Institute of Molecular Medicine, University of Lisbon
Coupling pre-mRNA processing to release from the site of transcription

10:15 – 10:30 **Pavel Hozak**
Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague
Nuclear myosin I is involved in transcription

10:30 – 11:00 *Coffee Break*

Session 3

Chair: **David Shore**

11:00 – 11:10 **Jana Vlasakova**
Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague
Histone deacetylase inhibitors suppress IFN-alpha-induced up-regulation of promyelocytic leukemia protein

11:10 – 11:30 **Gustav Ammerer**
Max F. Perutz Laboratories, University of Vienna
Stress-regulated transcription

11:30 – 11:50 **Francesc Posas**
Cell Signaling Unit, University Pompeu Fabra, Barcelona
Stress-regulated transcription by the SAPK Hog1

11:50 – 12:10 **Pavel Kovarik**
Max F. Perutz Laboratories, University of Vienna
Stat1 targets p38 MAPK-mediated changes in general transcription complexes to specific promoters

12:10 – 12:30 **Christoph Schüller**
Max F. Perutz Laboratories, University of Vienna
Activation of stress response genes as default response to loss of chromatin remodelling

12:30 – 14:00 *Lunch*

14:00 Guided tour of Mendel Museum (optional)

16:00 – 18:00 **Posters & Coffee**

18:00 *Dinner*

Session 4

Chair: **Maria Carmo-Fonseca**

19:00 – 20:00 Plenary Lecture
Kim Nasmyth
University of Oxford
How do cells hold sister chromatids together?

20:00 – 20:25 **Jan Ellenberg**
EMBL, Heidelberg
Functional dynamics of SMC complexes in living cells

20:25 – 20:50 **Jan-Michael Peters**
Institute of Molecular Pathology, Vienna
Regulation of sister chromatid cohesion in mammalian cells

Saturday 14 October

08:00 – 09:30 EuroDYNA Scientific Board Breakfast Meeting in Hotel

Session 5

Chair: **Jan-Michael Peters and Colin Logie**

10:00 – 10:30 Remarks and Conclusions from Board Meeting
Colin Logie, Jan-Michael Peters, Astrid Lunkes

10:30 – 10:55 **Niels Galjart**
Department of Cell Biology and Genetics Erasmus University, Rotterdam
Functional analysis of murine CTCF

10:55 – 11:20 **Colin Logie**
Nijmegen Centre for Molecular Life Sciences, University of Nijmegen
Nucleosomal arrays and the ATP-dependent chromatin remodelling complexes yRSC and dNuRD

11:20 – 11:45 *Coffee Break*

Session 6

Chair: **Herbert Lindner**

11:45 – 12:10 **Jean Thomas**
Department of Biochemistry, University of Cambridge, UK
Linker histones and their interactions with DNA and chromatin

12:10 – 12:35 **Marc Timmers**
Department of Physiological Chemistry, University of Utrecht
Mobility of TBP and TBP-complexes

12:35 – 13:00 **Adriaan Houtsmuller**
Department of Pathology, Erasmus MC, Rotterdam
Simultaneous FRAP and FRET reveal compartmentalisation of androgen receptor protein-protein interactions in living cells

13:00 *Lunch*

14:00 **Departure**

Speakers abstracts

Gustav Ammerer (AT)

Affiliation

Dept. of Biochemistry and
Molecular Cell Biology
University of Vienna, AT

Stress-regulated transcription

Arp4, an essential actin-related protein of budding yeast is an important subunit of at least three chromatin modifying complexes : NuA4, INO80 and SWR-C. They have been reported to be involved in the DNA repair and regulation of transcription. Many Msn2/Msn4 dependent genes were upregulated in arp4 thermosensitive (ts) mutant. Moreover, also the CCR-NOT complex was shown to be a negative regulator of Msn2/4 dependent transcription. We investigated whether Arp4 is directly involved in the repression of transcription of stress genes. We found that Arp4 binds to the promoter of HSP12 following heat shock and also observed dissociation of Arp4 from this promoter after stress release. This indicates that Arp4 is recruited to the promoters of stress genes only under stress conditions. The binding of Arp4 and Not5 (subunit of the CCR-NOT complex) following heat shock is dependent on the presence of Msn2. Interestingly, the association between Arp4 and HSP12, CTT1 open reading frames (ORFs) is much more pronounced than between Arp4 and the respective promoters. A similar effect could be observed for Not5 and the HSP12 locus. Taken together, these data tentatively suggest a connection between transcription elongation and recruitment of these repressive complexes.

Poster: Ludmila Paskova, Christoph Schueller, Andriy Petryshyn, Ulrike Wintersberger, Gustav Ammerer

Jiri Bartek (CZ)

Affiliation

Dept. of Cell Cycle and Cancer
Institute of Cancer Biology
Danish Cancer Society, DK

Centre for Genotoxic
Stress Research
Copenhagen, DK

Spatio-temporal organization of nuclear tumour suppressor proteins in response to DNA damage

Bartek J, Lukas C, Bekker-Jensen S, Bartkova J, Mailand N and Lukas J.

To protect the genome against adverse effects of DNA damage, surveillance pathways (checkpoints) coordinate cell cycle progression with DNA repair, thereby preventing diseases such as cancer. The lecture will summarize our recent data on the mechanistic basis and spatiotemporal control of the checkpoint pathways in nuclei of living mammalian cells. We have constructed a micro-laser unit that allows generation of distinct types of DNA lesions including DNA double-strand breaks and analysis of protein redistribution within seconds after DNA damage. When combined with genetics, biochemistry, and a variety of interactive photo-bleaching assays to study cells stably expressing GFP-labeled checkpoint proteins, this system allows quantitative assessment of the earliest molecular assemblies at the generated DNA lesions, as well as biological consequences of their deregulation. Examples of our real-time imaging of molecular trafficking inside the nucleus, dynamics of protein complexes at the sites of DNA damage, and a broader impact of such events in protection of genomic integrity will be presented.

Maria Carmo-Fonseca (PT)

Affiliation

Institute of Molecular Medicine
Faculty of Medical Sciences
University of Lisbon, PT

Coupling pre-mRNA processing to release from the site of transcription

We have previously shown that transcripts derived from human ß-globin genes containing splice site mutations are retained at the site of transcription. These transcripts failed to recruit components of the splicesome and of the Exon Junction Complex in vivo, suggesting a possible implication of these proteins in mRNA release. However, transcription of normal human ß-globin genes by a variant of RNA polymerase II lacking 21 CTD repeats supports normal pre-mRNA processing and EJC recruitment but causes retention of the mRNA at the site of transcription. Thus, Pol II appears to play a role in mRNA release independent of pre-mRNA processing. Furthermore, we observe that a human ß-globin gene containing a 5' splice site mutation that abolishes splicing is significantly less transcribed than the normal gene. Chromatin immunoprecipitation and run on assays suggest that the presence of the mutation leads to a defect in CTD phosphorylation and decreased density of active RNA pol II along the gene. Taken together our results reveal a complex interplay between pre-mRNA splicing, RNA pol II transcription and mRNA release from the site of transcription.

Jan Ellenberg (DE)

Affiliation

EMBL, Heidelberg, DE

Functional dynamics of SMC complexes in living cells

Restructuring chromatin into morphologically distinct chromosomes is essential for cell division, but the molecular mechanisms underlying this process are poorly understood. Condensin complexes have been proposed as key factors, although controversial conclusions about their contribution to chromosome structure were reached by different experimental approaches in fixed cells or cell extracts. Their function under physiological conditions still needs to be defined. Results: Here, we investigated the specific functions of condensin I and II in live cells by fluorescence microscopy and RNAi depletion. Photobleaching and quantitative time-lapse imaging showed that GFP-tagged condensin II bound stably to chromosomes throughout mitosis. By contrast, the canonical condensin I interacted dynamically with chromatin after completion of prophase compaction, reaching steady-state levels on chromosomes before congression. In condensin I-depleted cells, compaction was normal, but chromosomes were mechanically labile and unable to withstand spindle forces during alignment. However, normal levels of condensin II were not required for chromosome stability.

Cohesin is a multisubunit protein complex that links sister chromatids from replication until segregation. The lack of obvious cohesin-targeting-specific sequences on DNA, as well as cohesin's molecular arrangement as a large ring, has led to the working hypothesis that cohesin acts as a direct topological linker. To preserve the identity of sister chromatids, such a linkage would need to stably persist throughout the entire S and G2 phases of the cell cycle. Unexpectedly, cohesin binds chromatin already in telophase, and a large fraction dissociates from chromosomes during prophase in a phosphorylation-dependent manner, whereas initiation of anaphase requires proteolytic cleavage of only a small fraction of cohesin. These observations raised the question of how and when cohesin interacts with chromatin during the cell cycle. Here, we report a cell-cycle dependence in the stability of cohesin binding to chromatin. Using photobleaching and quantitative live-cell imaging, we identified several cohesin pools with different chromatin binding stabilities. Although all chromatin bound cohesin dissociated after a mean residence time of less than 25 min before replication, about one-third of cohesin was bound much more stably after S phase and persisted until metaphase, consistent with long-lived links mediating cohesion between sister chromatids.

Anna A. Friedl (DE)

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Radiobiological Institute
University of Munich, DE

Radiation-induced Chromatin Alterations: Application of Ion Microirradiation

Authors: A.A. Friedl, G.A. Drexler, S. Auer, I. Baur, S. Breitkopf, H. Strickfaden, S. Dietzel, T. Cremer, A. Hauptner, R. Krücken, V. Hable, C. Greubel, G. Dollinger.

While the repair of radiation-induced DNA lesions is performed in the context of chromatin structure and nuclear architecture, little is known about the influence of these parameters on the cellular response to DNA damage. Using the ion microbeam SNAKE at the Munich tandem accelerator, various types of experiments can be perforemd to investigate chromatin dynamics and alterations. After applying single ions in geometric patterns and immunoflurescence labeling of damaged regions using antibodies recognizing foci-forming proteins (e.g. gamma-H2AX), the stability of the pattern with incubation time after irradiation can be investigated. In non-synchronized HeLa cells small scale mobility can be seen which is compatible with constrained diffusion processes. After 24 h of incubation, the patterns appear rather conserved in some cells, while they have drastically changed in other cell, suggesting an influence of cell cycle phase on pattern stability. We currently investigate the influence of cell cycle in synchronized cells. In addition, by siRNA mediated knock-down of candidate genes we investigate which proteins may be responsible for the constraints in the mobility of damaged DNA. A detailed investigation of focus sizes revealed complex alterations after ion irradiation: Focus sizes initially increase, before the sizes reduces and then remains stable over 20 h.Preliminary data suggest that this pattern reflects chromatin alterations, since it can be modified by treatment with factors that inhibit histone modifying enzymes. Currently we investigate which types of histone modifications take place during the cellular response to DNA damage.

Niels Galjart (NL)

Affiliation

Dept. of Cell Biology
and Genetics
Erasmus University Medical
Centre, NL

Functional analysis of murine CTCF

The conserved zinc finger protein CTCF and its testis-specific paralogue BORIS have been implicated as key players in transcriptional regulation, the maintenance of epigenetic states and enhancer-blocking. A common function ascribed to these proteins is that of an insulator: a protein that can build chromatin environments which can target (or shield) specific genes towards (or from) the actions of enhancers or repressors. To investigate the in vivo functions of murine CTCF and BORIS, we have generated inducible knock out and GFP knock in alleles of both genes. Here, I will discuss our recent results obtained with mice carrying these alleles.

Adriaan B. Houtsmuller (NL)

Affiliation

Dept. of Pathology
Josephine Nefkens Institute
Erasmus University, NL

Simultaneous FRAP and FRET reveal compartmentalisation of androgen receptor protein-protein interactions in living cells

Steroid receptors (SRs) regulate gene expression in a ligand-dependent manner by binding specific DNA sequences. Binding of ligand induces a conformational change of the ligand binding domain (LBD), allowing interaction with coregulators containing LxxLL motifs. The androgen receptor (AR) preferentially interacts with coregulators containing LxxLL-related FxxLF motifs. The AR is regulated at an extra level by interaction of an FQNLF motif in the N-terminal domain (NTD) with the C-terminal LBD (N/C-interaction). Although it is generally recognised that these protein-protein interactions are essential for proper regulation of gene transcription by SRs, it is largely unknown where and when they are required. To investigate the spatio-temporal organisation of AR-coregulator and AR N/C interactions, we have performed simultaneous fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) measurements in single living cells expressing ARs double tagged with the yellow (YFP) and cyan (CFP) variants of the green fluorescent protein (GFP), at the N- and C-terminus respectively. We provide evidence that AR N/C-interactions occur predominantly when ARs are freely mobile in the nucleus, possibly to prevent unfavourable or untimely cofactor interactions. In contrast, AR-coregulator interactions occur preferentially when ARs are immobilized by DNA-binding.

This work was supported by grant DDHK 2002-2679 of the Dutch Cancer Society KWF.

Pavel Hozák (CZ)

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Dept. of Cell Ultrastructure
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Dept. of Biology of
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Institute of Molecular Genetics
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Histone deacetylase inhibitors suppress IFN-alpha-induced up-regulation of promyelocytic leukemia protein

J. Vlasáková, Z. Nováková, L. Janderová-Rossmeislová, P. Hozák, Z. Hodný.

Promyelocytic leukemia nuclear bodies (PML NBs), the structural domains of the eukaryotic cell nucleus, play a role in cancer and apoptosis, and their involvement in antiviral mechanisms mediated by interferons (IFNs) is proposed. IFNs dramatically increase the transcription of the PML gene. In this study, we have shown that the response of two structural PML NBs components, PML and Sp100, to interferon-alpha was suppressed in cells simultaneously treated with histone deacetylases (HDAC) inhibitors (trichostatin A, MS-275, SAHA, and sodium butyrate). Trichostatin A blocked the increase of PML NBs number and suppressed up regulation of PML mRNA and protein levels in several human cell lines and in normal diploid skin fibroblasts. Moreover, alpha-induction of IRF-1 was also inhibited by HDAC inhibitors, although incompletely. Analysis of cellular fractions did not show any defects in cytoplasmic-nuclear transport of STAT2, a component of transcription factor ISGF3 responsible for IFN-alpha/beta-induction. Moreover, chromatin immunoprecipitation with STAT2 antibody revealed binding of STAT2 to ISRE element of PML promoter after IFN-alpha-stimulation even in the presence of trichostatin A. These results indicate that deacetylation is a necessary event for full transcriptional activation of IFN-alpha-stimulated genes.

Torben Heick Jensen (DK)

Affiliation

Dept. of Molecular Biology [1]
University of Aarhus, DK

Dept. of Human Genetics [2]
University of Aarhus, DK.

Severely compromised transcription due to a 5' splice site mutation

Torben Heick Jensen[1], Christian Kroun Damgaard[1], Søren Kahns[2], Søren Lykke-Andersen[1], Anders Lade Nielsen[2], Jørgen Kjems[1].

Transcription and mRNA processing are closely coupled interdependent nuclear events. Examples of "downstream coupling" in which promoter- and/or RNA polymerase II-properties affect mRNA processing are quite well characterized, however mechanisms by which mRNA processing might affect transcription are poorly understood. We have used cell lines stably expressing HIV-1 or ?-globin mRNAs harboring wild-type or mutant 5' splice sites (5'ss). Surprisingly, a 5'ss mutation causes a marked decrease in steady state levels of mRNA in both contexts. Data suggests that this phenotype is due to inefficient recognition of the mutated 5'ss sequence by U1snRNP.

Attempts to define the mRNA degradation pathway for the "unstable" 5'ss-mutated RNA have been fruitless. In contrast, RNA-FISH and transcription run on analyses reveal an app. 10-fold transcriptional downregulation in splice site mutants. This result is confirmed by RNA polymerase II chromatin immuno precipitation analysis, which also indicates a transcription initiation/early elongation failure. Chromatin structure analysis shows significantly higher acetylation levels of histone 3, lysine 9 within the wild-type transgene compared to the mutant. Surprisingly, histone 3, lysine 9 methylation, normally associated with a repressed transcriptional state, is not significantly different between the two genes. Thus, the mechanism by which an abberantly processed transcript communicates with the transcription machinery, is transient and not associated with repressive chromatin methylation.

Leonie M. Kamminga (NL)

Affiliation

Netherlands Institute for Developmental Biology, NL

Piwi proteins and small RNAs in the zebrafish germline

Argonaute proteins comprise a highly conserved protein family, and are involved in a variety of RNA silencing phenomena in a diverse set of organisms. In short, they bind small RNA molecules, like siRNAs and miRNAs, and use these short RNAs as guides to find homologous target mRNAs. Subsequently, the expression of these targeted mRNAs is repressed. Based on sequence, the Argonaute proteins can be subdivided into two subfamilies, the Ago and Piwi subfamily. Piwi proteins are required for germline development in a diversity of organisms, like M. musculus, C. elegans, Drosophila, and Arabidopsis. In zebrafish one piwi homologue has been described, ziwi, and we have identified a second: zili. Like in other organisms, these genes are specifically expressed in the germline. In addition, we find that these genes are already expressed in the embryonic precursors of the germline: the primordial germ cells. We have generated mutant zili and ziwi zebrafish and we will describe their phenotype.

The mechanism how piwi proteins, and for that matter, zili and ziwi proteins act in RNA silencing is unknown. For instance, it is unclear which small RNAs and which proteins they bind to. In analogy to mice (Hannon pers, comm.), we have found that in the germline of the zebrafish a prominent class of approximately 30 nucleotide (nt) long RNAs is expressed. We find these RNAs in both the testis and ovaria of adult zebrafish and are unable to detect similar RNAs in other tissues. Interestingly, this 30nt species has not yet been found in ovaria of mice, most likely because of the different nature of oocyte development between mouse and zebrafish. Currently, we are cloning and sequencing this new class of 30nt RNAs in zebrafish and will present data regarding their origin and (sub)cellular localization. Furthermore, the potential interaction with these small RNAs with ziwi or zili proteins will be addressed.

Pavel Kovarik (AT)

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Dept. of Microbiology and Immunobiology
Max F. Perutz Laboratories
University of Vienna, AT

Stat1 targets p38 MAPK-mediated changes in general transcription complexes to specific promoters

Both interferon (IFN) and stress signals are required for full activation of immune responses. We and others have shown that the stress-activated p38MAPK increases transcription by Stat1, the key transcription factor of interferon signaling. Activation of Stat1 by IFNs is sufficient for transcription of IFN-stimulated genes. However, the transcription of many of these genes is increased by simultaneous activation of the stress-regulated p38 MAPK. In fact, our data demonstrate that in the context of several promoters the Stat1-mediated transcription is strictly dependent on p38 MAPK: Stat1 remains silent unless a co-stimulating p38 MAPK-mediated signal is present. By using the synergy of p38 MAPK and IFN/Stat1 we have identified novel IFN-regulated genes that play an important role in immune homeostasis. We have also evidence that p38 MAPK increases transcription by other Stat proteins as well indicating that the p38 MAPK/Stat synergy has a general role in gene transcription. The mechanism of the p38-mediated effects is still not understood. Our recent findings suggest that CDK kinases regulating RNA polymerase II activity are targeted to specific promoters in a Stat1- and p38 MAPK-dependent manner. In our model, Stat1 serves as a recruitment factor for these kinases ensuring that the stress/p38 MAP-induced transcriptional effects are channeled only to selected genes.

Francesc Posas (PT)

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Stress-regulated transcription by the SAPK Hog1

Eulàlia de Nadal, Glòria Mas, Meritxell Zapater, Núria Noriega, Alex Vendrell, Sergi Regot & Francesc Posas.

Exposure of yeast cells to increases in extracellular osmolarity results in the activation of the Hog1 MAP kinase. Activation of this MAP kinase is required to generate a set of osmoadaptive responses essential to survive under high osmolarity conditions. Adaptation to osmostress requires the induction of a large number of genes, which indicates the necessity to regulate several aspects of the cell physiology. Induction of gene expression is highly dependent on the presence of the MAP kinase, which suggests a key role for the HOG signaling pathway in the regulation of gene expression in response to osmostress. Several transcription factors are controlled by the MAPK, such as Sko1, Smp1, Hot1 and Msn2,Msn4, which regulate gene expression in response to stress. Interestingly, in response to stress, the MAPK controls several mechanisms related to transcription initiation; phosphorylation of transcription factors, chromatin modification and recruitment of PolII. In addition, recruitment of Hog1 to ORFs during elongation is critical for proper mRNA production in response to stress.

Christoph Schüller (AT)

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Dept. of Biochemistry
Factuty of Life Sciences
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Activation of stress response genes as default response to loss of chromatin remodelling

Ludmila Paskova, Christoph Schueller, Andriy Petryshyn, Eva Klopf, Ulrike Wintersberger, Gustav Ammerer.

Negative regulation of stress response genes occurs across ORFs: Arp4, an essential actin-related protein of budding yeast is an important subunit of at least three chromatin modifying complexes : NuA4, INO80 and SWR-C. They have been reported to be involved in the DNA repair and regulation of transcription. Many Msn2/Msn4 dependent genes were upregulated in arp4 thermosensitive (ts) mutant. Moreover, also CCR-NOT complex was shown to be the negative regulator of Msn2/4 dependent transcription. We investigated whether Arp4 is directly involved in the regulation of transcription of stress genes. We found that Arp4 binds to the promoter of HSP12 following heat shock and also observed dissociation of Arp4 from this promoter after stress release. This indicates, that Arp4 is recruited to the promoters of stress genes only under stress conditions. The binding of Arp4 and Not5 (subunit of the CCR-NOT complex) following heat shock is dependent on the presence of Msn2. Interestingly, the association between Arp4 and HSP12, CTT1 open reading frames (ORFs) is much more pronounced then between Arp4 and the respective promoters. A similar effect could be observed for Not5 and the HSP12 locus. Taken together, these data tentatively suggest a connection between transcription elongation and recruitment of these repressive complexes.

Jean O. Thomas (UK)

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Dept. of Biochemistry
University of Cambridge, UK

Linker histones and their interactions with DNA and chromatin

The general principles of chromatin structure are universally conserved in eukaryotes. The fundamental subunit is the nucleosome. This comprises 166 bp of DNA wound in two superhelical turns around an octamer of the four core histones and stabilised by one molecule of linker histone (H1 or, in chicken erythrocytes, H5), and a variable length of linker DNA. The 10 nm nucleosome filament, a repeating array of connected nucleosomes, is further folded into higher-order structure (the "30 nm filament"). Linker histones, together with core histone post-translational modifications, play a major role in determining the equilibrium between these more open and closed states, respectively, and thus in regulation of transcription and probably other chromosomal processes that are controlled by chromatin accessibility.

Linker histones have a dual role in chromatin: in sealing the two turns of DNA around the histone octamer, which is a property of the central globular domain of H1 and H5; and in controlling chromatin condensation through association of the long basic C-terminal (and possibly also the shorter N-terminal) tail with the internucleosomal DNA. The C-terminal tail is subject to cell-cycle-dependent (in the case of H1) and developmentally regulated (H5) phosphorylation at "-SPKK-" motifs, which generally reduces the affinity of the linker histone tail for DNA. There is no high-resolution structural information for how either the globular domain or the tails of linker histones interact with DNA or chromatin, although for the globular domain there is a reasonable model based on various lines of biochemical evidence.

Structural information on linker histones and their interactions will be reviewed. In our ESF-funded collaboration we aim to understand better the relationship between phosphorylation of linker histones and their structural and functional roles. Work in Innsbruck and Linköping is largely concerned with characterisation of cell-cycle-dependent phosphorylations and different linker histone subtypes, and with in situ interactions of linker histones, and in Cambridge with structural studies to understand how linker histones interact with DNA and chromatin and particularly how this is affected by phosphorylation.

Marc Timmers (NL)

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Dept. for Physiological Chemistry
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Mobility of TBP and TBP-complexes

H.Th. Marc Timmers, Florence Mousson, W.W.M. Pim Pijnappel, Marcel G.T. Winter, Folkert J. van Werven, Hetty A.A.M. van Teeffelen and Petra de Graaf.

The TATA-binding protein (TBP) is central to eukaryotic transcription and resides in different TBP-TAF complexes (SL1, TFIID, B-TFIID, TAC, and TFIIIB). Comparison of TBP-binding surfaces for TAF subunits of these complexes explains their mutually exclusive binding to TBP.

Little is known about stability of TBP-complexes and exchange of TBP and TAFs. To address this we applied different experimental strategies. First, we determined TBP-complex mobility in human cells by GFP-FRAP experiments. Secondly, we employed a SILAC-based proteomics approach to analyze TBP-complex subunit exchange in cell extracts. Thirdly, in yeast cells we analyzed global redistribution of TBP by ChIP-on-Chip upon transcriptional reprogramming.

The results of these approaches will be discussed.

Poster abstracts

Simon Bekker-Jensen (DK)

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Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks

Simon Bekker-Jensen, Claudia Lukas, Shizhou Liu, Fredrik Melander, Jiri Bartek and Jiri Lukas.

In response to DNA double strand breaks (DSBs), many proteins accumulate into microscopically discernible structures, known as ionizing radiation-induced foci (IRIF). This trait of the DNA damage response is highly conserved in evolution and is widely regarded to facilitate genomic integrity in cells exposed to DNA damage. However, surprisingly little is known about how DNA repair and checkpoint signalling processes benefit from the increased local concentration of certain factors. By combining DSB-generating laser microirradiation, immuno-staining with target-specific antibodies and high-resolution confocal microscopy, we provide evidence for several qualitatively distinct modes of focal accumulation of proteins at the DSB sites. First, the chromatin compartment marked by phosphorylated H2AX is occupied by checkpoint mediator proteins such as Mdc1 and 53BP1 together with the ATM kinase and its related complexes. These interactions are equally robust throughout the interphase. Second, proteins from the homologous recombination machinery such as BRCA2, FANCD2 and the entire ATR binding cascade are excluded from chromatin and accumulate in smaller focal structures. These “micro foci” are delineated by single stranded DNA, the resection of which is restricted to S and G2 phases of the cell cycle. A third class of DSB regulators, including Chk1, Chk2 and the last of the three main PI3K kinases, DNA-PK, do not show any focal accumulation. As we previously showed for Chk2, the interaction of some of these proteins with DSBs is likely too transient to manifest itself as cytologically discernible foci. Interestingly, a small group of key DSB regulators appear to simultaneously occupy several compartments. Thus, BRCA1 and Nbs1 were found to accumulate into repair as well as chromatin associated foci, likely reflecting the well-established dual repair and signalling roles of these two proteins. Together, these results help sub-classify DSB regulators according to their precise residence sites after DNA damage, and provide a framework to validate, predict, or even exclude, functional interaction between DSB regulators. As an example of this, we combined the above approach with biochemical techniques to dissect the roles of Claspin and TopBP1 in the ATR pathway. We found Claspin, like Chk1, to have a pan-nuclear distribution following laser damage, while TopBP1 tightly co-localized with ATR in micro foci. Biochemical experiments confirmed that TopBP1 has a general role in supporting ATR phosphorylations, while Claspin is restricted to supporting activation of Chk1.

Fiona Gardiner (UK)

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High resolution imaging of cohesin and condensin structures by FESEM

During mitosis, the cohesin protein complex mediates cohesion between sister chromatid arms, concentrating at centromeric regions until the metaphase-anaphase transition. At this transition the Scc1 subunit of cohesin is cleaved by the cysteine protease separase and centromeric cohesin dissociates from chromosome arms, initiating sister chromatid separation. Additionally, the condensin protein complex also associates with chromatin during mitosis and is involved in chromatin condensation. This complex is known to associate with chromatin from early prophase and is required for the correct localisation of chromatin associated proteins including topoisomerase II and INCENP.

While previous immunofluorescence studies have localised both complexes to specific chromosomal regions, our studies however pinpoint specific cohesin and condensin subunits in situ in combination with established kinetochore and chromosomal markers. High resolution imaging of these complexes in Hela cells was achieved using FESEM (Field Emission Scanning Electron Microscopy) in combination with Immuno-gold Labelling. The identification and visualisation of cohesin and condensin subunits on compacting and compacted chromatin enables further insight into the structure and function of these complexes.

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Investigating the functions of CTCF in mice

CTCF is a highly conserved gene. The protein CTCF binds to divergent sequences of DNA and other proteins via the central 11 zinc-finger binding domain. There are many functions attributed to CTCF in the cell ranging from mediating transcriptional activation or repression to the regulation of imprinted gene expression. There is no consenus site for CTCF to bind DNA, indeed CTCF is reported to even bind both methylated and unmethylated DNA. Interestingly, CTCF has been identified as a so called enhancer-blocking protein, and remains the only known vertebrate protein with the ability to act as an insulator. Recently, we have shown CTCF to be important in the formation of chromatin loops and histone modifications at the b-globin locus in mice. Using a conditional CTCF knock-out mouse, we are currently investigating the function of CTCF during T-cell development. We show that CTCF is crucial for cell division and proliferation but not differentiation of T-cells as they progress along the T-lineage. Interestingly, T-helper cell development seems to be impaired in the absence of CTCF. Here we present data demonstrating the importance of CTCF during T-cell development.

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Targets and functions of RNA-directed DNA methylation and Pol IVb in Arabidopsis thaliana

RNA-directed DNA methylation, which is one of several RNAi-mediated pathways in the nucleus, appears to be highly elaborated in the plant kingdom. Genetic screens in our lab have identified several plant-specific proteins required for this process: DRD1 (defective in RNA-directed DNA methylation) is a plant-specific, putative SWI/SNF-like chromatin remodelling protein. DRD2 and DRD3 (renamed NRPD2a and NRPD1b) are subunits of a plant-specific RNA polymerase termed Pol IV. Recent work has focused on identifying endogenous DNA sequences that are methylated and silenced by these proteins and histone modifications associated with this pathway, with the aim of understanding the natural functions of RNA-directed DNA methylation and Pol IV in plants.

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Quantitative in vivo analysis of protein mobility using the software Tropical

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Background
Fluorescent protein (FP) chimeras are commonplace in cell biology to study subcellular protein localization and protein dynamics in live cells. This has led to an increasing number of reports using fluorescence recovery after photobleaching (FRAP). Many previous publications were mainly concerned with estimating the diffusion coefficient only or, in some cases, relative recovery half times have been published. It has been shown that relative recovery half times do not allow cross-study comparisons of molecular mobilities [1]. Taking into account inhomogenous protein distribution can further improve the precision of parameter estimation [2, 3]. To this end, we developed a simulation software for reaction-diffusion systems that allows estimation of the diffusion coefficient and kinetic reaction rates based on FRAP image series [4].

Methods
We have selected a number of nuclear proteins to generate their respective GFP-fusions. Intranuclear mobilities are estimated from FRAP image series using our in-house developed simulation software “Tropical”. This software implements a reaction-diffusion model with finite-differences discretization, Runge-Kutta fourth-order ODE solver and Levenberg-Marquardt optimization. The accessible parameters are the diffusion coefficient and association/dissociation rates in protein-protein interactions.

Results
Tropical allows users to describe protein interactions in terms of reaction equations. Based on these partial differential equations the software calculates a solution. Reaction and diffusion parameters are varied until a good agreement between the simulated and the recorded image series is achieved. Researchers without programming knowledge can use this tool to estimate the diffusion coefficient and reaction parameters as long as a reaction equation is known. Tropical has been applied to an artificial (simulated) reaction-diffusion model and to B23-GFP stably expressed in A431 squamous cell carcinoma cells to estimate D and koff [4].

Significance
In light of the current demand for in vivo kinetic data both for drug screening and simulation of signal transduction networks quantitative annotation of protein-protein and protein-DNA interactions can be a bottleneck [5, 6]. Tropical can be seen as a general tool for providing such data. FRAP is a well established technique and widely applicable on standard hardware. The focus on image series as input data therefore allows a large number of labs to perform a detailed analysis of molecular mobility.

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Negative regulation of stress response genes occurs across ORFs.

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Arp4, an essential actin-related protein of budding yeast is an important subunit of at least three chromatin modifying complexes : NuA4, INO80 and SWR-C. They have been reported to be involved in the DNA repair and regulation of transcription. Many Msn2/Msn4 dependent genes were upregulated in arp4 thermosensitive (ts) mutant. Moreover, also CCR-NOT complex was shown to be the negative regulator of Msn2/4 dependent transcription. We investigated whether Arp4 is directly involved in the regulation of transcription of stress genes. We found that Arp4 binds to the promoter of HSP12 following heat shock and also observed dissociation of Arp4 from this promoter after stress release. This indicates, that Arp4 is recruited to the promoters of stress genes only under stress conditions. The binding of Arp4 and Not5 (subunit of the CCR-NOT complex) following heat shock is dependent on the presence of Msn2. Interestingly, the association between Arp4 and HSP12, CTT1 open reading frames (ORFs) is much more pronounced then between Arp4 and the respective promoters. A similar effect could be observed for Not5 and the HSP12 locus. Taken together, these data tentatively suggest a connection between transcription elongation and recruitment of these repressive complexes.

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Stat1 targets p38 MAPK-mediated changes in general transcription complexes to specific promoters

Both interferon (IFN) and stress signals are required for full activation of immune responses. We and others have shown that the stress-activated p38MAPK increases transcription by Stat1, the key transcription factor of interferon signaling. Activation of Stat1 by IFNs is sufficient for transcription of IFN-stimulated genes. However, the transcription of many of these genes is increased by simultaneous activation of the stress-regulated p38 MAPK. In fact, our data demonstrate that in the context of several promoters the Stat1-mediated transcription is strictly dependent on p38 MAPK: Stat1 remains silent unless a co-stimulating p38 MAPK-mediated signal is present. By using the synergy of p38 MAPK and IFN/Stat1 we have identified novel IFN-regulated genes that play an important role in immune homeostasis. We have also evidence that p38 MAPK increases transcription by other Stat proteins as well indicating that the p38 MAPK/Stat synergy has a general role in gene transcription. The mechanism of the p38-mediated effects is still not understood. Our recent findings suggest that CDK kinases regulating RNA polymerase II activity are targeted to specific promoters in a Stat1- and p38 MAPK-dependent manner. In our model, Stat1 serves as a recruitment factor for these kinases ensuring that the stress/p38 MAP-induced transcriptional effects are channeled only to selected genes.

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Regulation of ribosome biogenesis by Sfp1 in budding yeast

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Regulation of ribosome biogenesis is essential to cell growth, since in optimally growing cells more than 50% of total Pol II transcription is dedicated to ribosomal protein genes (RP genes) and genes controlling ribosome biogenesis (Ribi genes). Therefore transcription of these genes is regulated in a very precise manner by various nutritional and stress signals. TOR and Ras/PKA are two main signaling pathways linking nutritional signals to regulation of RP and Ribi gene transcription. One of the downstream effectors of TOR and PKA in S. cerevisiae is Sfp1. Sfp1 is a putative transcription factor that has been shown to regulate the transcription of very large number of RP and Ribi genes in a TOR1- and PKA-dependent manner. How TOR1 and PKA regulate Sfp1 is currently unknown and it is also unclear how Sfp1 regulates the expression of RP and Ribi genes.

To learn more about Sfp1 function we used a one-step TAP purification to identify in vivo interaction partners. Notably, we found that TOR1 and KOG1, components of the rapamycin-sensitive TOR Complex 1 (TORC1), bind to Sfp1, suggesting that Sfp1 might function directly downstream of TOR1 kinase. However, in an in vitro kinase assay using purified TORC1 and recombinant Sfp1 we could not detect Sfp1 phosphorylation. We are currently determining how the TORC1-Sfp1 interaction is regulated by different growth and stress conditions. We have also begun a phosphopeptide analysis of Sfp1 to determine if it is phosphorylated in vivo in a TOR-dependent manner. We have identified two putative phosphorylation sites in Sfp1 that seem to be absent in rapamycin treated cells. We are testing if mutations of these sites interfere with the function of Sfp1 and if these sites are targets for either TOR1 or PKA.

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The small-molecule inhibitor BI 2536 reveals novel roles of polo-like kinase 1 in mitotic entry and maintenance of bipolar spindles

Cyclin-dependent kinase 1 (Cdk1), Aurora kinases and Polo-like kinase 1 (Plk1) are essential regulators of mitosis and have been extensively characterized to understand mitotic mechanisms, and as potential targets for cancer therapy. Studies of Cdk1 and Aurora kinases have been greatly facilitated by small-molecule inhibitors, but for Plk1 few if any selective inhibitors have been reported so far.

We characterized the cellular effects of a novel small molecule, BI 2536, that inhibits Plk1 with high potency (IC50 = 0.83 nM) and selectivity. In both human cancer derived HeLa and telomerase immortalized RPE1 cells BI 2536 mimics the effects of RNA interference (RNAi) mediated Plk1 depletion, such as delayed mitotic entry, prometaphase arrest, defects in centrosome maturation and spindle assembly. Unlike RNAi, BI 2536 causes these phenotypes with complete penetrance and rapid onset, which enabled us to study previously controversial or unknown functions of Plk1. Cells that enter mitosis in the presence of BI 2536 fail to form microtubule asters in prophase, and polymerize mitotic microtubules only after nuclear envelope breakdown. These microtubules assemble into monopolar spindles but do not stably attach to kinetochores, leading to spindle checkpoint induced arrest. When cells with established bipolar spindles are treated with BI 2536, spindle poles lose focus, kinetochores detach from microtubules and the checkpoint is reactivated. Thus, Plk1 is not only required for assembly but also for maintenance of bipolar spindles. Furthermore, we show that BI 2536 inhibits degradation of the APC/C inhibitor Emi1 but does not delay cyclin A proteolysis, indicating that Emi1 destruction is not essential for activation of APC/C in early prometaphase.

Together, we show that BI 2536 is a potent and specific inhibitor of Plk1 in cultured human cells that can be used as a versatile tool in mitosis research and, potentially, in cancer therapy.

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Histone H1 Phosphorylation occurs site-specifically during interphase and mitosis

H1 histones, isolated from logarithmically growing and mitotically-enriched human lymphoblastic T-cells (CCRF-CEM), were fractionated by reversed phase and hydrophilic interaction liquid chromatography, subjected to enzymatic digestion and analyzed by reversed phase high performance liquid chromatography, amino acid sequencing and mass spectrometry. During interphase the four H1 subtypes present in these cells differ in their maximum phosphorylation levels: histone H1.5 is tri, H1.4 di-, and H1.3 and H1.2, only mono-phosphorylated. The phosphorylation is site-specific and occurs exclusively on serine residues of SP(K/A)K motifs. The phosphorylation sites of histone H1.5 from mitotically-enriched cells were also examined. In contrast to the situation in interphase, at mitosis there were additional phosphorylations, exclusively at threonine residues. Whereas the tetraphosphorylated H1.5 arises from the triphosphosphorylated form by phosphorylation of one of two TPKK motifs in the C terminus, namely Thr137 and Thr154, the pentaphosphorylated H1.5 was the result of phosphorylation of one of the tetraphosphorylated forms at a novel non-consensus motif at Thr10 in the N-terminal tail. Despite the fact that histone H1.5 has five (S/T)P(K/A)K motifs, all of these motifs were never found to be simultaneously phosphorylated. Our data suggest that phosphorylation of human H1 variants occurs non-randomly during both interpase and mitosis and that distinct serine- or threonine-specific kinases are involved in different cell-cycle phases. The order of increased phosphorylation and the position of modification might be necessary for regulated chromatin decondensation, thus facilitating processes of replication and transcription as well as of mitotic chromosome condensation.

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Ifh1: the key player at ribosome protein gene promoters

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The rate of protein synthesis, and hence the availability of ribosomes, is a central factor in determining cell growth. Currently unknown mechanisms assure that equimolar amounts of rRNA and each of the ribosomal proteins (RPs) are produced in the cell. This process is exquisitely coordinated with the synthesis of hundreds of different non-ribosomal proteins, which participate in ribosome biogenesis.

In budding yeast, coordinated expression of the RP genes is primarily accomplished at the transcriptional level. Two factors, Fhl1 and Ifh1, bound to the active RP promoters, are key components of the dynamic transcriptional network that controls RP gene expression. The association of Ifh1 to RP gene promoters depends on its interaction with the forkhead transcription factor Fhl1. While Fhl1 is constitutively bound to ribosomal genes, Ifh1 binding seems to be tightly controlled and correlates with the activation of ribosomal genes.

Loss of Ifh1 from the RP promoters has been attributed to competition from the Ifh1-like molecule Crf1 that enters the nucleus as a result of inhibition of the TOR pathway.

We will show that, at least in some genetic backgrounds, Crf1 has no apparent role in the control of RP gene expression, as Ifh1 leaves the promoters in the absence of Crf1. Our data suggests that some other mechanism is responsible for the disappearance of Ifh1 from the RP genes. We propose that Ifh1, and not Crf1, is in fact the key regulator in the RP gene transcription network.

A functional analysis of different regions and residues of the Ifh1 protein as well as a study of the interactions between Ifh1 and other factors involved in activation of the RP genes, such as PKA and Rap1 will be presented. Our data will help to understand how the interactions between Ifh1 and other RP promoter-associated factors leads to the transcriptional activation of these genes.

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Ifh1 regulation of ribosomal protein gene expression

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Ribosome biogenesis, which provides the protein synthesis capacity necessary for mass accumulation, is the major driving force underlying cell growth. Rapidly growing yeast cells must devote approximately half of all RNA polymerase II transcription to ribosomal protein genes, producing up to 2,000 ribosomes per minute. This rapid production of ribosomes requires an enormous investment of energy, and it is thus essential that cells appropriately regulate ribosome biogenesis in response to both growth and other environmental signals. Our lab and others have recently obtained evidence for a working model in Saccharomyces cerevisiae (Baker's yeast) in which under favorable growth conditions, the transcriptional activator Ifh1 is recruited to ribosomal protein (RP) gene promoters through an interaction with the constitutively bound factor Fhl1. The presence of Ifh1 at the promoter correlates with the activation of RP genes. When conditions become unfavorable for cell growth, Ifh1 leaves the RP promoters resulting in a rapid downregulation of RP gene expression. Through collaboration with Prof. Gustav Ammerer (University of Vienna), we have used mass spectrometry to identify changes in the phosphorylation pattern of Ifh1 under these different conditions. Based on these results, we are currently using site directed mutagenesis and Chromatin immuno-precipitation techniques to identify which phosphorylation events on Ifh1 are responsible for its recruitment to RP gene promoters through the interaction with Fhl1.

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Investigating the structural role of linker histone phosphorylation

Linker histones (H1, and in chicken erythrocytes H5) play a major role in maintaining higher-order chromatin structure by interacting with the linker DNA between nucleosomes to permit the condensation of chromatin. This role may be regulated by reversible phosphorylation of the H1 C-terminal tails at "–SPKK-" motifs. Currently there is no detailed structural information on the interaction of either the central globular domain or the tails of H1 with DNA, or on the effect of phosphorylation on the tail/DNA interaction. We aim to study these interactions using four-way junction DNA. This is a preferred DNA substrate for linker histone globular domains, probably because its juxtaposed duplexes mimic the entry/exit points of DNA in the nucleosome; the junction arms should provide binding sites for the N- and C-terminal tails.

As a necessary pre-requisite for the structural studies, we have established conditions for stoichiometric phosphorylation of H1 and H5 with recombinant CDK2/cyclin A at all the "-SPKK-" sites in the C-terminal tail, and in the case of H5 an additional (non-SPKK) site in the N-terminal tail, as demonstrated by mass spectrometry. We are also investigating C-terminal tail truncations of recombinant H5, giving products with different numbers of -SPKK- sites, which should be useful in the interaction studies.

We have shown by gel-retardation analysis that both native H1 and H5, and recombinant truncations of H5, can form discrete complexes with four-way junctions under conditions approximating as closely as possible those necessary for NMR. We are therefore embarking on NMR experiments to study directly (a) the interaction of the central globular domain of the linker histones with junction DNA, and (b) the interaction of the C-terminal tail with the junction arms and how this is regulated by phosphorylation.

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Determining the in vivo targets and the role of SF1 (splicing factor 1) in the nucleus and cytoplasm.

Julian Pakay and Angela Krämer.

SF1 functions in nuclear pre-mRNA splicing by binding to the intron branch point sequence (BPS) near the 3' splice site, where it facilitates the binding of U2AF65 to the adjacent polypyrimidine tract, which in turn allows recruitment of the U2 snRNP to the BPS. Although SF1 was initially characterised as being an essential component for pre-spliceosome formation, recent studies have demonstrated that its role in splicing is kinetic rather than obligate. For example, splicing of several endogenous and reporter pre-mRNAs was unaffected by RNAi-mediated depletion of SF1, although it is required for cell viability1.
This essential component of SF1 function remains elusive. It is possible that SF1 is required for the splicing of a specific subset of pre-mRNAs such as those with sub-optimal splice sites2. Another possibility is that SF1 functions in a process other than splicing. Heterokaryon experiments have demonstrated that SF1 continually shuttles between the nucleus and the cytoplasm. However, the mechanism and significance of this shuttling remains unknown.
To gain insight into an essential function(s) of SF1 we have employed the cross-linking and immunoprecipitation technique 3 and isolated in vivo pre-mRNA targets of SF1 from nuclear and cytoplasmic fractions of HeLa cells.

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Analyses of linker histone - chromatin interactions in situ

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The linker histones, commonly referred to as H1, are involved in the formation and maintenance of the higher order structure of the chromatin fiber and most likely also in epigenetic modulation of gene expression. In mammals, this family consists of eight subtypes, H1.1-H1.5, H1t, H1° and H1oo. The highly specialized isoforms H5 (in avian species) and H1° accumulate in some terminally differentiated cells. In avian and amphibian erythrocytes, expressing H5 and H1° respectively, these proteins have been linked to cessation of cell proliferation and condensation of chromatin. We have studied the interaction between linker histones and chromatin in a number of cell types, using 4´6-diamidino-2-phenylindole (DAPI) as an indirect cytochemical probe for linker histone affinity in situ, in combination with high performance capillary electrophoresis and reverse-phase high performance liquid chromatography. Significant differences were detected between some cell types. The results show that linker histones have a substantially higher affinity for chromatin in mature chicken erythrocytes than in frog erythrocytes. This difference may possibly be explained by the high content of arginine-rich H5 in chicken erythrocytes. Our results also indicate that the affinity decreased in differentiating frog erythrocytes, showing the lowest affinity in terminally differentiated cells with highly condensed chromatin. Furthermore, in cultured human fibroblasts the linker histones showed a relatively high affinity for chromatin. However, in highly condensed metaphase chromosomes, the affinity was significantly lower compared to interphase cells. We have also analyzed linker histone affinity for chromatin in H1-depleted fibroblasts reconstituted with purified linker histones. The results show that the exogenous linker histones were bound with slightly lower affinity than the native ones and that in vitro phosphorylated linker histones were bound with substantially reduced affinity. Our results also indicate a lack of correlation between linker histone affinity and chromatin condensation.

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Dynamics of Translesion synthesis polymerases in living cells

DNA damage is a continual problem for all cells. Cells have evolved a series of repair systems to deal with the majority of the injuries that can occur to the genetic material. However these systems are often slow and incomplete, and a second line of defence is achieved via a number of tolerance mechanisms that allow DNA to be replicated despite the presence of unrepaired damage. One of those mechanisms, called DNA translesion synthesis(TLS), utilizes specialized DNA polymerases to bypass a damaged template before reestablishing normal replication. In mammalian cells two of the major players in TLS are DNA polymerases poleta and poliota.

Studies using indirect immunofluorescence have shown that these polymerases colocalize with PCNA in replication factories forming clearly visible foci in the nucleus. When DNA replication is stalled by DNA damage, PCNA molecules associated with the stalled forks become mono-ubiquitinated. Poleta and poliota can bind to ubiquitinated PCNA via ubiquitin binding domains and are thereby recruited to the stalled replication forks to carry out TLS. While recent evidence has elucidated how TLS works and is regulated, very little is known about the in vivo dynamics of these polymerases. In this work we have established a number of cell lines stably expressing GFP tagged versions of pol and pol and we have taken a closer look at how they behave in living cells by using confocal microscopy and studying their mobility by Fluorescent Recovery after Photobleaching (FRAP). In contrast to PCNA, the focal component of poleta and poliota seems to be very dynamic with a very low residence time, indicating that only a small percentage of the protein present in the focus is actually engaged in the bypass mechanism. Moreover in absence of any damage, differences could be detected between the mobility of poleta and poliota possibly indicating a different mode of action of the two polymerases.

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Simultaneous FRAP and FRET reveal compartmentalisation of androgen receptor protein-protein interactions in living cells

Steroid receptors (SRs) regulate gene expression in a ligand-dependent manner by binding specific DNA sequences. Binding of ligand induces a conformational change of the ligand binding domain (LBD), allowing interaction with coregulators containing LxxLL motifs. The androgen receptor (AR) preferentially interacts with coregulators containing LxxLL-related FxxLF motifs. The AR is regulated at an extra level by interaction of an FQNLF motif in the N-terminal domain (NTD) with the C-terminal LBD (N/C-interaction). Although it is generally recognised that these protein-protein interactions are essential for proper regulation of gene transcription by SRs, it is largely unknown where and when they are required. To investigate the spatio-temporal organisation of AR-coregulator and AR N/C interactions, we have performed simultaneous fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) measurements in single living cells expressing ARs double tagged with the yellow (YFP) and cyan (CFP) variants of the green fluorescent protein (GFP), at the N- and C-terminus respectively. We provide evidence that AR N/C-interactions occur predominantly when ARs are freely mobile in the nucleus, possibly to prevent unfavourable or untimely cofactor interactions. In contrast, AR-coregulator interactions occur preferentially when ARs are immobilized by DNA-binding.

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Nuclear RNA quality control by coupled activity of Pap2 and exosome complexes

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In the yeast nucleus, different types of noncoding RNA molecules can be subjected to degradation as a consequence of changes in growth conditions or of incorrect processing. The main activity responsible for this degradation is the nuclear exosome. We have previously characterized a polyadenylation complex consisting of Pap2p (formerly called Trf4p), Air1p, Air2p and Mtr4p that can efficiently stimulate the exosome activity in vitro. TAP-tagged versions of Pap2/Trf4p and Rrp6p, a protein present exclusively in the nuclear form of the exosome, were used to prepare affinity-purified complexes. The exosome (Rrp6-TAP) was further purified by gel filtration and the proteins in the active fractions were identified by mass spectrometry. They contained most of the known subunits of the nuclear exosome complex as well as Imd3p, Ski1p, few polypeptides of the nuclear import machinery, Sen1, a component of the Nrd1 complex.
A thorough analysis of the catalytic properties of the exosome activity upon stimulation with the Pap2-TAP fractions confirmed previous predictions that multiple rounds of polyadenylation are needed at the 3' end to initiate digestion of the body of incorrectly folded tRNA molecules. The catalytically inactive mutant of Pap2p (DXD) failed to induce the RNA digestion by the exosome and degradation was also inhibited in the presence of the non-hydrolyzable ATP homolog cordycepin triphosphate. Furthermore, the end-products of the digested RNAs have been analyzed by enzymatic probing and thin-layer chromatography.
Furthermore, our recent assays with exosome fractions on short structured substrates suggested that in yeast, RNAs access the catalytic centre in different way than it has been reported for the archeal exosome. Currently, we are testing the hypothesis that core exosomes acquire specific activities by the association with different co-factors.

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The CSB protein: More than “just” a transcription–coupled repair factor?

Cockayne syndrome is a disorder characterized by developmental and neurological abnormalities, photosensitivity and premature ageing. Apart from its proposed role as a coupling factor between a stalled RNA polymerase and the repair machinery, additional activities have been suggested for the CSB protein. It has been proposed that CSB can act to push an RNA polymerase over a certain sequence that significantly decreases the transcription elongation rate. Such a sequence could be a natural pausing site, but could also contain DNA damages that block transcription. To test this hypothesis we have constructed DNA templates containing different lesions at defined positions and studied the transcription response to DNA damage in the absence or presence of CSB.

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Live cell dynamics of mammalian nucleotide excision repair

Nucleotide Excision Repair (NER) is a versatile repair process that eliminates a variety of helix-distorting injuries, including UV-induced DNA damage. Its biological significance is underscored by the severe clinical consequences (i.e. UV-induced skin cancer and premature aging) associated with inherited NER-deficient syndromes. NER is a multi-step process requiring ~ 30 polypeptides. Previous studies revealed an intimate contact between NER and transcription. Moreover, NER utilizes different replication factors for the repair synthesis stage. Despite detailed knowledge of each of the separate mechanisms, little is known about the dynamic interplay and regulation between these processes. Key components of each process were tagged with GFP and expressed in mammalian cells. Clones were selected that stably express functional fusion proteins at physiological relevant levels. Spatio-temporal distribution, live cell protein dynamics, protein-protein interactions and reaction kinetics of NER, transcription and replication factors were determined by using time-lapse imaging and different variants of FRAP (fluorescence recovery after photobleaching). These studies suggest a stochastic and versatile 'on the spot assembly' model, driven by diffusion and random collision. Initiation of these processes is mainly determined by the availability of affinity or target sites under changing conditions.

Like many other cellular processes, DNA repair is in part controlled by adjusting steady-state levels of critical factors via transcriptional regulation. To implement the effect of damage-induced gene expression on modulation NER-efficiency we started to generate the next series of GFP-tagged NER factors by knocking in the fusion protein at the endogenous gene locus. By gene targeting we have generated mouse-models that express GFP-tagged XPB (one of the components of TFIIH) at different levels allowing kinetic measurements with varying concentrations of TFIIH.

In addition, regulation of nuclear process, including DNA repair, is often accomplished by post-translational protein modifications, such as ubiquitylation. Recently, we identified a DNA damage-induced increase of an immobile nuclear ubiquitin pool. This process appeared to be strictly dependent on functional NER and likely occurs post-incision. Subsequent analysis revealed that the main target of this ubiquitylation event is histone H2A, which becomes mono-ubiquitylated. H2A ubiquitylation is a late event in DNA repair following assembly of the excision complex. We suggest that the chromatin remodeling by this damage-induced mono-ubiquitylation of H2A is a component of the signaling cascade initiated in response to UV damage.

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Histone deacetylase inhibitors suppress IFN-alpha-induced up-regulation of promyelocytic leukemia protein

Promyelocytic leukemia nuclear bodies (PML NBs), the structural domains of the eukaryotic cell nucleus, play a role in cancer and apoptosis, and their involvement in antiviral mechanisms mediated by interferons (IFNs) is proposed. IFNs dramatically increase the transcription of the PML gene. In this study, we have shown that the response of two structural PML NBs components, PML and Sp100, to interferon-alpha was suppressed in cells simultaneously treated with histone deacetylases (HDAC) inhibitors (trichostatin A, MS-275, SAHA, and sodium butyrate). Trichostatin A blocked the increase of PML NBs number and suppressed up-regulation of PML mRNA and protein levels in several human cell lines and in normal diploid skin fibroblasts. Moreover, IFN-alpha-induction of IRF-1 was also inhibited by HDAC inhibitors, although incompletely. Analysis of cellular fractions did not show any defects in cytoplasmic-nuclear transport of STAT2, a component of transcription factor ISGF3 responsible for IFN-alpha/ß-induction. Moreover, chromatin immunoprecipitation with STAT2 antibody revealed binding of STAT2 to ISRE element of PML promoter after IFN- alpha-stimulation even in the presence of trichostatin A. These results indicate that deacetylation is a necessary event for full transcriptional activation of IFN-alpha-stimulated genes. Noticeably, HDAC inhibitors are tested as potent anti-cancer reagents in different types of solid tumor cell lines and in hematopoietic transformed cell line, several of them are in initial phases of clinical trials, therefore their suppressing effect on IFN- alpha pathway should be taken into account.

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Interphase cohesion and DNA double strand break repair

From yeast to man, sister chromatids are held together from their synthesis during S-phase until they are segregated during mitosis. This sister chromatid cohesion depends on a protein complex called cohesin. We have started to investigate the role of cohesin during interphase in human cultured cells. In particular, we are interested in the function of cohesin and sister chromatid cohesion in the repair of DNA double-strand breaks (DSB). We showed that cohesin and cohesin regulators such as sororin are required for DSB repair in G2-phase, and that depletion of the cohesin subunit Scc1 affects the arrest of cells that normally occurs in response to DSBs. We are currently trying to understand how the cohesin complex participates in the DNA damage checkpoint.

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