Workshop on
Cell Biology & Microscopy

… for phD students and young postdocs

Mo 17th – Th 20th
May 2010

Organisers: S. Diekmann and C. Cremer

Youth hostel Bad Herrenalb
(Ferienheim Aschenhuette)

Abstractbook

Workshop on Cell Biology & Microscopy

Monday 17.05. 2010	Tuesday 18.05.2010	Wednesday 19.05.2010	Thursday 20.05.2010
	Session 1 9:00 h V. Döring 9:40 h K. Klement 10:20 h break 10:45 h R. Janoštiak 11:25 h V. Dion	Session 3 9:00 h M. Bombouskova 9:40 h S. Marthandan 10:20 h break 10:45 h O. El Sayed	take down posters Departure
	12:30 h – 13:30 h Lunch	12:30 h – 13:30 h Lunch	
Arrival	Poster Session 14:00 h – 15:30 h	Posters, free	
Check in			
	Session 2 15:30 h G. Best 16:10 h M. Gunkel	Session 4 15:30 h F. Arnhold 16:10 h A. Scharf	
Put-up posters	16:50 h R. Kaufmann		
18:30 h – 19:30 h Dinner	18:30 h – 19:30 h Dinner	18:30 h – 19:30 h Dinner	
Opening Lecture 20:00 h St. Diekmann		Closing Lecture 20:00 h C. Cremer	

According to demand, we can have discussion rounds on selected topics (like for example: properties of fluorescent proteins, the Abbe limit, application of new microscope techniques, comparison of biochemical with microscope techniques, etc.).

in vivo analysis of the MCC – APC/C complex

Volker Döring

The anaphase promoting complex/ cyclosome (APC/C) is a stable multiprotein complex that triggers the progression from metaphase to anaphase during mitosis. This E3-Ligase consists of at least 12 subunits and initiates sister chromatid-separation by ubiquitination of securin, the repressor of separase. Seperase becomes free and is then able to degrade the sister chromatides connecting cohesion. In case of missing or improper spindle-attachment of only one kinetochore to the spindle apparatus a stop signal is generated by the spindle assembly checkpoint. This checkpoint is a surveillance mechanism that inhibits the APC/C by assembly of the mitotic checkpoint complex (MCC). The MCC is formed by BubR1, Bub3, Mad2 and Cdc20. Only when all kinetochores are properly attached to the spindle microtubules the MCC becomes deactivated and Cdc20, a potent mitotic activator of the APC/C, can bind and activate the APC/C. Failure in this system result in premature sister chromatid separation which in turn may lead to aneuploidy, cellular senescence or even cancer. Although the architecture of the human and yeast APC/C is elucidated, current knowledge of the interaction between the MCC, the outer kinetochore and the APC/C is limited. Also little is known about the time resolved distribution of those complexes. We therefore are interested in the identification of the interaction partners between MCC, the outer kinetochore and the APC/C. Also we want to study dynamics and molecular interactions. For our *in vivo* analysis we started to localize fluorescence-tagged proteins of the MCC and APC/C respectively in living human cells. To investigate stability, composition, interaction and the dynamics of all the proteins we will perform FRAP, FRET, FCS and FLIM analysis. Therefore we tagged each protein with C- and N-terminally to various fluorescence proteins (GFP, YFP, Cerulean, mCherry, mRFP). Beside our *in vivo* studies we want to analyse protein-protein-interactions by *in vitro* analysis. Therefore we also tagged each protein of the MCC and the APC/C with Y2H expression-tags.

Isoform-specific chromatin binding of heterochromatin protein-1 in living cells

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Heterochromatin maintenance is crucial for proper chromosome segregation, clonal inheritance of cell identity and the regulation of gene expression. Although architecturally stable, heterochromatin has to be flexible to cope with biochemical events on DNA. One possible mechanism underlying functional flexibiliy of heterochromatin relies on heterochromatin protein (HP1) populations with different residence times [1-3]. To further dissect individual contributions of HP1 isoforms to heterochromatin maintenance we have assessed the dynamics of HP1α, HP1β, and HP1γ by live-cell microscopy and mathematic modeling.

Our results indicate that a considerable fraction of all three HP1 isoforms diffuses freely within the nucleoplasm. The chromatin-binding population is divided into two major classes, a fast one with residence times of approx. 5 sec, and a slower one with residence times in the minute range. HP1γ exhibits more stable binding in euchromatin than HP1α and HP1β. In heterochromatin the residence time of the slow fraction of HP1β is 3 to 5-fold higher than for HP1α and HP1γ. The apparent diffusion coefficients of all HP1 isoforms as assessed by FCS and RICS in euchromatin (D = 1.3 $\mu m^2 s^{-1}$) and heterochromatin (D = 0.6 $\mu m^2 s^{-1}$) are almost identical. We also show that loss of the chromo domain or the chromoshadow domain renders HP1β unable to form the slow exchanging population, indicating that the domains act in concert for proper chromatin binding. The hinge region does not contribute to chromatin binding at all.

Our observations for the first time indicate isoform-specific chromatin dynamics of HP1 proteins in living cells probably reflecting some individual duties on chromatin.

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Biological significance of Tyr12 phosphorylation in p130CAS

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CAS is a major tyrosine phosphorylated protein in cells transformed by v-*crk* and v-*src* oncogenes. CAS plays an important role in invasiveness and metastasis of Src-transformed cells. In breast cancer patients, high CAS levels are associated with higher recurrence of disease, poor response to tamoxifen treatment, and lower overall survival. In nontransformed cells, CAS localizes to focal adhesions and undergoes tyrosine phosphorylation within its large substrate domain in response to integrin-mediated adhesion. Recently, a new phosphorylation on tyrosine 12 (Y12) within the ligand-binding hydrophobic pocket of CAS SH3 domain was found to be enriched in Src-transformed mouse embryonic fibroblasts (MEFs).

To study importance of Y12 phosphorylation we have prepared phosphomimicking Y12E and non-phosphorylable Y12F mutations of full-length CAS. We have found that phosphomimicking mutation leads to loss of binding of CAS to FAK and PTP-PEST and to decrease of FAK and Paxillin phosphorylation. Expression of Y12E CAS in MEFs and Src-transformed sarcoma cells caused a great decrease in localization of CAS to focal adhesions but the localization to podosomes-type adhesions was not affected. Furthermore, expression of Y12E CAS in Src-transformed CAS-/- MEFs greatly enhanced the rate of cell migration into a monolayer wound and invasion in 3D collagen. Taken together our data suggest an important role of Y12 phosphorylation on regulation of CAS-mediated signaling.

Dynamics of double-strand breaks: Implications for repair

Vincent Dion, Véronique Kalck, and Susan M. Gasser.

Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland.

A single double-strand break (DSB) can kill a cell. Therefore, cells have evolved a plethora of repair and signalling proteins, which converge to DSBs to trigger repair and checkpoint arrest. Despite the significant progress in the roles of specific proteins in DSB repair, the organization of the repair process in the nuclear space is poorly understood. Recently, using budding yeast as a model system, we have shown that irrepairable breaks are recruited to the nuclear periphery whereas physiologically relevant spontaneous breaks that occur during S-phase appeared to prefer an internal nuclear location. Here, we aim to define the mechanism by which deferent types of damaged DNA will be recruited or retained to the appropriate subnuclear zone and how chromatin movement is regulated during the process of repair. We used high precision fluorescence microscopy to visualize and quantify DSBs location and movement in living cells. We find that spontaneous breaks marked by Mre11-YFP, Rad52-YFP, YFP-Rad51, and Rad54-YFP are internal, providing strong evidence that repair of DSBs via homologous recombination is concentrated to the nuclear interior. In addition, the movement of repair foci formed by break markers that are recruited early in the repair process are as fast as an intact chromatin locus, but the movement eventually drops and the repair foci become highly constrained. Interestingly, Rad51, a protein involved in homology search and strand invasion, is required for proper localization of Rad52-YFP foci and for the drop in movement of Rad52-YFP tagged breaks. Rad52-YFP tagged breaks that occur in G1 – as the result of checkpoint inhibition through caffeine treatment – do not have the sister chromatid necessary for strand invasion, but nonetheless see their movement decrease similar to S-phase DSBs where the sister is available. Thus, the drop in chromatin movement during DSB repair is not merely a by-product of the repair process, nor is it controlled by checkpoint kinases. We are now considering the hypothesis that chromatin movement is controlled by remodelling of chromatin at sites of DNA breaks and that chromatin movement may contribute to homology search.

Dual Color Localization Micrsocopy

Manuel Gunkel

The Dual Color Localization Microscopy (2CLM) presented here is based on the principles of Spectral Precision Distance Microscopy (SPDM) with conventional fluorochromes under special physical conditions. This technique allows us to measure the spatial distribution of single fluorescently labeled molecules in entire cells with an effective optical resolution comparable to macromolecular dimensions. Here, we describe the application of the 2CLM approach to the simultaneous imaging of cellular structures using two fluorochrome types distinguished by different fluorescence emission wavelengths. The capabilities of 2CLM for studying the spatial organization of the genome in the mammalian cell nucleus are demonstrated for the relative distributions of two chromosomal proteins labeled with GFP and mRFP1 domains. The 2CLM images revealed quantitative information on their spatial relationships down to length-scales of 30 nm.

Statistical Analysis of Membrane Protein Distributions Using Localisation Microscopy

Rainer Kaufmann, Patrick Müller, Michael Hausmann, Christoph Cremer

In recent years various techniques of sub-diffraction limit light microscopy have been established. One of these is represented by localisation microscopy, which not only provides a structural resolution down to the 20 nm range, but also individual information about each detected molecule. This enables novel approaches for investigation of the spatial arrangement of proteins and statistical analysis of their distribution on the single molecule level. We could characterise the clustering of receptor proteins on the plasma membrane and use this as a sensitive tool for differentiation between different breast cancer cell lines and healthy cells of a mamma biopsy.

Electron Microscopy on Isolated Membrane Sheets as a Tool for Studies of Membrane-bound Activation Events

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Ultrastructural analysis of isolated plasma membrane (PM) sheets using transmission electron microscopy (TEM) is a powerful technique for understanding the topography and organization of PM-bound signaling molecules. Specific probes are used to label receptors and signaling proteins on the extracellular and cytoplasmatic side of native membrane sheets prepared from live cells. Cells can be stimulated in various ways before isolation that gives an opportunity to follow up changes during cell activation. We use mast cells as a model for studying immunoreceptor signaling. These cells have long been recognized for their role in the genesis of allergic inflammation, and more recently for their participation in innate and acquired immune responses. Activation of mast cells is triggered by binding of a multivalent antigen (Ag) to specific IgE anchored to the high affinity receptors for IgE (FcεRI). This step initiates a signaling cascade that results in formation of multicomponent signaling complexes (signalosomes) on PM, further propagation of the signal and subsequent degranulation. The earliest known biochemical step that occurs after binding of multivalent ligand to the IgE-FcεRI complexes is tyrosine phosphorylation of the receptor subunits. However, the exact molecular mechanism of this phosphorylation is incompletely understood. We tested the hypothesis that changes in activity and/or topography of protein tyrosine phosphatases (PTPs) could play a major role. We found that exposure of rat basophilic leukemia cells or mouse bone marrow-derived mast cells to PTP inhibitors [H_2O_2 or pervanadate (Pv)], induced phosphorylation of the FcεRI subunits, similarly as FcεRI triggering. Interestingly, and in sharp contrast to FcεRI-induced activation, neither H_2O_2 nor Pv induced any changes in the association of FcεRI with detergent-resistant membranes (DRMs). For detail analysis of FcεRI distribution we focused on the topography of FcεRI on membrane sheets as detected by electron microscopy. Membrane sheets were isolated from nonactivated or activated mast cells using simple procedure (1-7). We have confirmed our previous data that exposure of the cells to multivalent antigen induced formation of FcεRI aggregates as detected by an antibody recognizing cytoplasmic portion of the FcεRI chain. In contrast, we did not find any receptor clusters after exposure of the cells to Pv. In cells stimulated with Pv, H_2O_2 or antigen, enhanced oxidation of active site cysteine was detected in several PTPs. Unexpectedly, most of oxidized phosphatases bound to the plasma membrane were associated with cytoskeleton-like structures. Experiments with biotin-labeled phalloidin, followed by gold-labeled streptavidin, and electron microscopy analysis showed that oxidized phosphatases colocalize with actin cytoskeleton. Based on these and other data we propose that down-regulation of enzymatic activity of PTPs and/or changes in their accessibility to the substrates play a key role in initial tyrosine phosphorylation of the FcεRI and other multichain immune receptors.

References:

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Does Oxidative stress contribute to T-cell senescence?

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Age-related deterioration and dysregulation of T cell function, termed 'immunosenescence', may lead to increased mortality and morbidity in humans through greater susceptibility to infections and diseases. Previous research from the group suggested that oxidative stress may play a role in the immunosenescence process, causing genomic instability and cell cycle delay and arrest.

The aim of this research program is to investigate the effect of oxidative stress and intracellular redox potential on T cell function *in vitro* and *ex vivo* using peripheral blood mononuclear cells (PBMCs) and T cell clones (TCCs) from normal and aged donors. The effect of different concentrations of antioxidants (Ebselen & N-Acetyl Cysteine) on the proliferative capacity & life span of the cells, changes in redox tone (GSH:GSSG ratio) relative to oxidative DNA damage and ageing and the mechanisms behind T cell dysfunction will be evaluated.

The results of this investigation revealed that optimum concentrations of these antioxidants revealed significant ROS scavenging capacity in T cell samples *ex vivo* irrespective of the age group of the donors, but in TCCs, only when supplemented from a young *in vitro* age. In this latter situation, age related changes in T cell physiology example: reduced DNA repair capacity, heat shock response and an accumulation of bimolecular damage may have contributed to these findings. Furthermore, the impact of these antioxidants on other senescence markers could be investigated.

Use of new fluorescent proteins for *in vivo* studies of human kinetochore proteins

Osama El-sayed, Christian Hoischen and Stephan Diekmann

Enhanced green fluorescent protein (eGFP) is a standard protein marker in life cell imaging studies. Its ability to generate a visible internal fluorophore makes it a perfect protein marker. In our lab we are using a variety of fluorophores that differ in the wavelength of their fluorescent light (eGFP, Cherry, Cerulean, YFP, *etc.*) to study the properties of proteins of the human kinetochore *in vivo*.
Recently new fluorescent proteins have become available like fluorophores of new wavelength (plum, Tag-RFP-T, turquoise*)* and a photoconvertable fluorescent protein (Eos) and a photoactivable fluorescent protein (PA-GFP).
We have started to generate vectors that harbour the information for a protein of interest attached to a PA-GFP. Initial experiments have shown that the new PA-GFP is a powerful tool to investigate the dynamics of the proteins of the human kinetochore in a time dependant manner in living cells.
In the near future we will also introduce other new fluorophores to our lab so that we are able to elucidate the assembly and function of the human kinetochore with a variety of life cell imaging techniques.

What happens to nuclear architecture when transcription is off?

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Since the primary sequence of the human genome is resolved the key question that emerged is how and when different gene programs are realized in one organism. To answer this question it is important to understand the integration of genome function into the architectural framework of the cell nucleus. RNA polymerase I (RNAP I) transcribes ribosomal RNA genes in the nucleolus, a defined subnuclear structure. Subtoxic concentrations of the xenobiotic mercury chloride specifically inhibit RNAP I-dependent transcription [Chen and von Mikecz, 2000]. As a consequence the nucleolar protein fibrillarin redistributes into nucleoplasmic clusters where it is degraded by the proteasome. Additionally, mercuric chloride induces an up to threefold increase of PML bodies in number and size. This is surprising since actinomycin D, which inhibits both, RNAP I- and II-dependent transcription, effects a decrease of the number of PML bodies. The nuclear envelope, centromeres and splicing speckles remain unchanged after mercury chloride treatment. Here, we will discuss further systematic microscopic analysis of subnuclear structures and chromatin under RNAP I off conditions.

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The role of proteasomal proteolysis in the transcription cycle

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The transcription cycle can be subdivided into three separate steps: initiation, elongation and termination. It is essential for the cell to control switching on and off genes at all times, therefore transcription is regulated during each of these steps. A possible regulatory mechanism is provided through the nuclear ubiquitin-proteasome-system (nUPS). Proteins are tagged with polyubiquitin chains via reaction with E1, E2 and E3 enzymes, are recognized by the 19S proteasomal regulatory complex, unfolded and finally channelled into the 20S core particle for proteolysis [Schwartz und Ciechanover, 2009]. In mammalian cells the proteolytic activity of nuclear proteasomes is organized in distinct foci [Rockel et al., 2005]. These focal proteolytic centers, detected via proteasome-dependent degradation of a model substrate into fluorescent peptides, appear irrespective of fixation or cell type and are not substrate specific. Polyubiquitinated proteins localize in approximately 29 proteolytic foci that are distributed throughout the nucleus. Chromatin profiling shows that active proteasomal proteolysis is not localized in heterochromatic regions of the nucleus, but is clearly visible in the euchromatin. Nuclear proteolysis enables on site degradation of transcriptionassociated proteins. Hence, regulation of transcription might occur through changes in the local concentration of RNA polymerases, transcription factors, activators, and regulators. Thus, the dynamic contribution of proteasomedependent proteolysis to global and local transcription will be discussed.

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Driftanalysis and Correction-Software for Localization Microscopy (SPDM)

Daniel Paech

Mechanical drift is an undesirable effect that occurs during microscopic measurements. To achieve nanoscale resolution in light microscopy it is essential to measure and, if needed, to correct the positions of detected signals. Without correction of mechanical drifts biological structures are smeared out. This yields to invalid results of quantitative analysis.

In the presented work I used fluorescent microspheres with 100 nm diameter to quantify the drift during measurements within typical duration times. An algorithm that automatically corrects the position of every signal within each frame is used as a tool for retracting the complete image.