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Cover image: Cryo-EM map of the scaffold structure of the lower eukaryotic nuclear pore as determined by subtomogram averaging (Beck et al., Nature 2007).
Electron optical density is shown isosurface rendered as seen from the cytoplasm; the inner and outer nuclear membranes are shaded in darker blue.
A new executive board

The members of the German Society for Cell Biology were asked to vote for a new executive board and three advisory board members earlier this year. The elected candidates all accepted their elections at the Annual Meeting 2012 in Dresden. With Oliver Gruss (Heidelberg) as chief operating officer and Klemens Rottner (Bonn) as secretary, two newcomers joined the executive board. The former chief operating officer Ralph Gräf (Potsdam) is the new vice president. The elected advisory board members are Doris Wedlich (Karlsruhe), Volker Gerke (Münster) and Eckhart Lammert (Düsseldorf). It is up to me as the new president of the German Society for Cell Biology to thank the DGZ members for their support.

We are dedicated scientists and it is our aim to continue the modernisation of the German Society for Cell Biology into an active, open minded and highly interacting scientific community. I also would like to thank the retired/retiring presidents Reinhard Fässler (Martinsried) and Harald Hermann (Heidelberg) who paved the way to put a new complexion on the German Society for Cell Biology. This became most obvious in the outstanding international meetings organized by the DGZ in the last years and the novel lay outs of our newsletter "Cell News" and web page. The new executive board considers it as a major task for the next years to further vitalize and unify the German cell biology community. A first step in this direction will be a joint Annual Meeting together with the German Society of Developmental Biologists (GfE) in Heidelberg 2013.

Annual Meeting 2012 in Dresden

The Annual Meeting 2012 took place in March in Dresden. The organizers Elisabeth Knust, Ewa Paluch and Marino Zerial (all of the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden) had set up a terrific programme and I would like to thank them in the name of the DGZ for organizing this outstanding conference. Most current trends in cell biology such as modelling, frontiers in microscopy, cytoskeleton mechanics or therapeutic delivery were addressed. Every plenary session and symposium had highly recognized leaders of the field as speakers, with Nobel laureate Günter Blobel (New York) highlighting the programme.

This years Annual Meeting has reached a scientific quality, which can not be topped. The attendance however was a bit disappointing. Altogether only around 400 participants were in Dresden. I guess it is a matter of all of us, who attended this exceptional meeting, to spread the word within the cell biology community that there is a meeting in Germany, which is of highest international standard and which provides a platform for scientific discussions, exchange and networking and should be used as a tool to interconnect the German cell biologists.

"Heißer Herbst"

Inspired by the former president Reinhard Fässler and put forward by his successor Harald Hermann, the German Society for Cell Biology since 2010 supports a novel meeting format, the "International Meetings". These special interest meetings cover a current cell biological hot spot. Topics in the past were imaging cell migration, the spider’s web: how microtubules organize cellular space, actin dynamics and physics of cancer. The new executive board, as one of its first actions, has implemented the International Meetings into the annual schedule of the DGZ. For the upcoming years the German Society for Cell Biology will support two International Meetings per year, one in spring and one in autumn.

Scientists who are planning to organize a meeting with an emphasis on a cell biological topic are hereby encouraged to contact the German Society for Cell Biology and ask for support.

In autumn we will have three International Meetings: the “2nd International Meeting on Actin Dynamics” (Regensburg) in September, the “3rd Physics of Cancer” meeting (Leipzig) in November and the meeting on “Molecular Concepts in Epithelial Differentiation, Pathogenesis and Repair” (Leipzig) in November. Last but not least, in September the German Society for Cell Biology will have its traditional annual Young Scientist Meeting in Jena, with a very attractive programme entitled “Cell Biology Shapes the Embryo”. All the meetings have very excellent speakers and further information is provided at the DGZ web page (www.zellbiologie.de).

Eugen Kerkhoff
SYMPOSIUM ANNOUNCEMENT

International Meeting of the German Society for Cell Biology
3rd Symposium Physics of Cancer (POC)
November 1st-3rd, 2012 in Leipzig, Germany

Organizers
Claudia T. Mierke (University of Leipzig),
Josef A. Käs (University of Leipzig),
Sarah Köster (University of Göttingen) and
Harald Herrmann (DFKZ Heidelberg)

The analysis of physical properties of cancer cells during the malignant progression of neoplasms is an emerging field in current cancer research, cell biology and biophysics. Several recent findings in this novel field revealed that biomechanical properties of cancer cells promote tumor growth and their migration through the human body including transendothelial migration, connective tissue invasion and extracellular matrix remodeling. Hence, biomechanical properties become the focus of recent research studies: For example, the actin cortex of cancer cells is much softer and supports fast cell growth and subsequently enhances cell division. Although the actin cortex softens, the cancer cells are still able to withstand high pressures exerted from their microenvironment which then supports tumor growth into the adjacent connective tissue. In turn, elements of the cancer cell’s cytoskeleton are pronounced which results in an overall stiffening of the tumor. In addition, stiffer cancer cells are able to generate increased contractile forces in order to move forward in a three-dimensional microenvironment. Finally, these new insights may contribute to understand how and why certain cancer cells possess the ability to invade, transmigrate and metastasize.

The topics of Physics of Cancer are:
• Biomechanics (Biopolymers, Networks, Rheology, Cytoskeleton, Cell Shape, Microenvironment)
• Forces, Cell Motility, Adhesion (Assembly of Focal Adhesions, Molecular Motors, Cell Division)
• Oncology (Metastasis, Transendothelial Migration)

Registration fee
220 € for all applicants (professors, post-docs, doctoral students)
140 € for students and DGZ members

The number of participants is limited. All applicants are invited to submit an abstract to apply for a short talk (limited number only!) and/or poster contribution.

Application deadline: September 30th, 2012

Please visit for further information and registration: www.uni-leipzig.de/poc/2012
Protokoll der Mitgliederversammlung 2012 der Deutschen Gesellschaft für Zellbiologie e.V. (DGZ)

Versammlungsort:
Congress Center Dresden,
Raum: Konferenzraum 3
Versammlungstag: Donnerstag, 22. März 2012
Beginn: 13:15 Uhr, Ende: 13:50 Uhr

Anwesend sind insgesamt 22 Teilnehmer: 19 Mitglieder und 3 amtierende Vorstandsmitglieder: Prof. Dr. Harald Herrmann-Lerdon (Präsident), Prof. Dr. Ralph Gräf (Geschäftsführer) und Prof. Dr. Eugen Kerkhoff (Sekretär).

Zum Protokoll der letzten Mitgliederversammlung werden keine Änderungs- oder Ergänzungswünsche geäußert.

Der Präsident der DGZ Prof. Dr. Harald Herrmann-Lerdon eröffnet die Versammlung mit einer kurzen Begrüßung der anwesenden Mitglieder.

TOP Geschäfts- und Kassenbericht
Der Geschäftsführer Prof. Dr. Ralph Gräf trägt den Kassenbericht des vergangenen Jahres 2011 vor und erläutert die Finanzlage der Gesellschaft anhand der Einnahmen- und Ausgaben-Bilanz:

TOP Entlastung des Vorstandes
Der Antrag zur Entlastung des Vorstandes wird eingebracht und der Vorstand wird ohne Gegenstimme und mit einer Enthaltung entlastet.

TOP Jahresbericht des Präsidenten
Der Präsident Prof. Dr. Harald Herrmann berichtet über die Aktivitäten der DGZ im Jahr 2011 und betont ausdrücklich die Bedeutung der Interaktion der Zellbiologie mit der Biophysik. Es wurden folgende Veranstaltungen durchgeführt:

• 34. DGZ-Jahrestagung am 30.03.-02.04.2011 in Bonn, organisiert von Dietter Fürst und Walter Witte
• DGZ/FEBS Workshop „The Spider’s Web: How microtubules organize cellular space“ am 29.06.-01.07.2011 in Potsdam, organisiert von Ralph Gräf und Manfred Schliwa

Harald Herrmann kündigt an, dass die Jahrestagung 2013 zusammen mit der Gesellschaft für Entwicklungsbiochemie (GBF) in Heidelberg durchgeführt wird.

Es wird darüber abgestimmt, ob in Zukunft auch eine DGZ/GBF-Doppelmitgliedschaft möglich ist und dem Antrag wird zuge stimmt.

BILANZ 2011
Einnahmen/Ausgaben-Zusammenstellung

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Summe der Einnahmen: 160.502,30
Guthaben am 31.12.2010: 223.139,84

| DGZ: | 155.469,25 |
| Werner Risau Preis: | 67.670,39 |

Summe der Ausgaben: 236.423,23
Guthaben am 31.12.2011: 147.218,71

DGZ: 84.378,66
Werner Risau Preis: 62.840,05

Die Einnahmen und Ausgaben waren am 31.03.2012 in Heidelberg durch die beiden Kassenprüfer Prof. Dr. Marie-Christine Babauville und Prof. Dr. Hans-Georg Mannhein geprüft und für richtig befunden worden.
TOP DGZ-Wahl 2012-2014
Es werden die Ergebnisse der DGZ-Wahl für die Amtsperiode 2012-2014 bekannt gegeben. Es waren 134 Stimmen ausgegangen, davon waren 133 gültig und 1 ungültig.

Vorstand: Zum neuen Präsidenten wurde Prof. Dr. Eugen Kerkhoff gewählt (122 Ja-Stimmen, 6 Nein-Stimmen, 4 Enthaltungen, keine Angaben: 1), zum neuen Vizepräsidenten Prof. Dr. Ralph Gräf (121 Ja-Stimmen, 5 Nein-Stimmen, 3 Enthaltungen, keine Angaben: 4), zum neuen Geschäftsleiter Prof. Dr. Oliver Gruss (118 Ja-Stimmen, 3 Nein-Stimmen, 8 Enthaltungen, keine Angaben: 4) und zum neuen Sekretär Prof. Dr. Klemens Rottner (115 Ja-Stimmen, 3 Nein-Stimmen, 11 Enthaltungen, keine Angaben: 4).

Ergänzungswahl Beirat: Prof. Dr. Volker Gerke (116 Ja-Stimmen, 1 Nein-Stimme, 12 Enthaltungen, keine Angaben: 1), Prof. Dr. Eckhard Lammert (107 Ja-Stimmen, 3 Nein-Stimmen, 18 Enthaltungen, keine Angaben: 5, Gegenvorschlag: Oliver Daumke), Prof. Dr. Doris Wedlich (115 Ja-Stimmen, 3 Nein-Stimmen, 11 Enthaltungen, keine Angaben: 4, Gegenvorschlag: Ralph Gräf).

Kassenprüfer: Prof. Dr. Marie-Christine Daubauvalle (122 Ja-Stimmen, 1 Nein-Stimme, 8 Enthaltungen, keine Angaben: 2), Prof. Dr. Hans-Georg Mannherz (118 Ja-Stimmen, 1 Nein-Stimme, 9 Enthaltungen, keine Angaben: 5).

Ergänzungswahl Preisjury: Dr. Frank Schnorrer (107 Ja-Stimmen, 3 Nein-Stimmen, 17 Enthaltungen, keine Angaben: 6, Gegenvorschlag: Wieland Huttner), Prof. Dr. Sabine Werner (121 Ja-Stimmen, 3 Nein-Stimmen, 9 Enthaltungen, keine Angaben: 0), Prof. Dr. Walter Witke (109 Ja-Stimmen, 2 Nein-Stimmen, 17 Enthaltungen, keine Angaben: 5, Gegenvorschlag: Walter Nickel).
Alle Kandidaten nehmen die Wahl an.

TOP Verschiedenes
Der Präsident fragt, ob es noch Fragen seitens der Mitglieder gibt. Da es keine Meldungen gibt, schließt der Präsident die Versammlung.

Prof. Dr. Harald Herrmann-Lerdon (Präsident und Versammlungsleiter)
Prof. Dr. Eugen Kerkhoff
(Sekretär und Protokollführer)
Morphogenesis of tissues

Jörg Großhans

A longstanding and central question in developmental biology is the generation of the shapes of tissues and organs during development. Studies in many species and developmental stages have described a great diversity of morphogenetic processes. By applying the toolboxes of molecular biology and modern microscopy allowing live imaging in high resolution to analysis of these morphogenetic processes, it appears that a limited and conserved set of modules controlling cell behaviour underlie tissue morphogenesis. A recurring module is apical constriction by contractile cortical actin-myosin filaments mediating invagination and folding of tissues, for example (Sawyer 2010). Pulsed contractions specifically at the apical cortex of actin-myosin filaments linked to the plasma membrane lead to an isotropic reduction in apical surface area (Martin 2009). The well-known players of microfilament dynamics, such as Rho signalling cascades, myosin motors, F-actin regulators, and of cell junctions, such as Cadherin complexes are involved in temporally and spatially controlled manner. Cell migration is another well-studied morphogenetic module. To achieve proper tissue organisation, solitary cells or groups of cells migrate from their origin to a distant destination. Prominent examples are the directed migration of the cells forming the germline (Tarbashevich 2010) or cells from the neural crest stereotypically migrating to a number of defined destinations within the embryo (Theveneau 2012). Underlying this migration behaviour, we find processes that have been studied by cell biologists in vitro, such as ameobid movement, and the well-known molecules controlling cytoskeletal dynamics, cell polarity and membrane organisation (Kardash 2010, Rorth 2011). The reduction of complex tissue behaviour to few well defined modules opens up this research to any cell biologist who is eager to put one’s favourite cell biological process into a physiological context. Although developmental biologists have benefited from the methods and mechanistical insights of cell biologist and visa versa for a long time, due to recent technological developments especially in microscopy and live imaging, rapid progress is expected at this disciplinary interface leading to mechanistic insight into longstanding problems.

On the tissue level morphogenesis is genetically defined leading to stereotypic cell behaviour according to cell fate. This does not mean however, that the behaviour of individual cells is defined at any given moment. Within a tissue a great variation of individual cell behaviour is observed. For example the mesodermal cells of Drosophila embryos contract their apical actin-myosin not simultaneously but rather in a dynamic salt-and-pepper pattern (Martin 2009). In crowded tissues, individual cells are extruded, despite having the same genetically defined fate (Marinari 2012). These observation imply that mechanisms of self-organisation are involved in coordination of the behaviour of individual cells within a given tissue. A challenge is to understand how the individual variations in timing and extent of cell behaviours give rise to a uniform collective behaviour (Lye 2011, Bosveld 2012)

This year’s young investigator meeting “Cell biology shapes the embryo” from September 20th to 22nd in Jena will provide a forum for discussion of topics at the interface of cell biology and developmental biology, including molecular mechanisms of cell polarity, cell migration, cell-cell adhesion. In addition to these processes on a cellular level, molecular and cellular mechanisms underlying tissue behaviour such as tissue movement, elongation, growth, and mechanics will be presented and discussed. A number of prominent experts in this field (please see the programme) will introduce their research topics and present their latest findings. In addition there will be plenty of time for short presentations and a poster session allowing active involvement and interaction with the invited speakers for all participants of the meeting.

References:


Figure: Cell shape changes defined by Rho signalling. Optical cross section of a fixed Drosophila embryo (about 150 μm) at the onset of gastrulation stained for RhoGFPs (green), Arm/B-catenin (red) and DNA (blue). Note the redistribution of RhoGFPs and Arm in the ventral cells, controlling apical constriction and cell shape changes that finally lead to invagination of the mesoderm anlage.
Prof. Dr. Jörg Großhans
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Institute of Biochemistry and Molecular Cell Biology
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37077 Göttingen
E-mail: jgrossh@gwdg.de

since 2009  Professor of Biochemistry, Univ. Göttingen,
2002-2008  Independent research group at the ZMBH Heidelberg (Emmy- Noether programme of the DFG)
1997-2001  Postdoc with E. Wieschaus, Univ. Princeton, USA
1993-1996  Doctoral work with C. Nüsslein-Volhard, Max-Planck-Institut Tübingen
1987-1993  Study of Biochemistry (Diplom) Univ. Tübingen and Univ. North Carolina, Chapel Hill, USA

13th DGZ Young Scientist Meeting
“Cell Biology shapes the Embryo”
September 20-22, 2012, Jena
Organizers: Jörg Großhans and Doris Wedlich

Master or Ph.D. students, postdocs and young group leaders are invited to apply for participation with an abstract. Participants will present their work as a poster or a short talk (selected by the abstracts).

Registration fee:
EUR 120,00 for regular participants
EUR 90,00 for students
Free for DGZ members
Registration includes accommodation and meals.

Application:
Your application (in one single WORD file and the file name should be your last and first name) should include:
– Name and contact details (e-mail address and full postal address)
– Status (DGZ member, student or regular participant)
– Abbreviated CV, max. 1 page
– ABSTRACT, max. 1 page
– incl. title, author(s) and affiliation(s)
– type: Arial, 12 p
– 1,5 lines spacing

Please send your application file by e-mail to the DGZ Office at dgz@delfz.de

The number of participants is limited.

Application Deadline:
August 10th, 2012

If you have any questions, please contact the DGZ Office at dgz@delfz.de
Scientific Programme

Thursday, September 20

until 14.00 Arrival of the speakers and participants
14:00 - 14:05 Welcome Address

Session I

14:05 - 14:15 Introduction by the organizers
14:15 - 14:55 Eyal Schejter (Rehovot): Actin cables guide polarized secretion in tubular organs
14:55 - 15:35 Michel Labouesse (Illkirch): How mechanical forces drive morphogenesis
15:35 - 16:15 Giorgio Scita (Milan): Endocytic control of the plasticity of cell migration
16:15 - 16:45 Coffee Break
16:45 - 17:00 Short talk 1 selected from the abstracts
17:00 - 17:15 Short talk 2 selected from the abstracts
17:55 - 18:05 Short talk 3 selected from the abstracts
18:05 - 18:45 Ray Keller (Charlottesville): The Mechanobiology of Gastrulation: Four Forces of Blastopore Closure
18:45 - 20:00 Get together and Dinner Reception
20:00 - 22:00 Poster Session

Friday, September 21

Session II

08:55 - 09:00 Introduction by the organizers
09:00 - 09:40 Erez Raz (Münster): Motility and directed migration of primordial germ cells in zebrafish
09:40 - 10:30 Pernille Rorth (Singapore): Guiding collective cell migration
10:30 - 10:45 Short talk 4 selected from the abstracts
10:45 - 11:00 Short talk 5 selected from the abstracts
11:00 - 11:30 Poster Session / Coffee Break
11:30 - 12:10 Doris Wedlich (Karlsruhe): Startling cadherin functions in collective migration
12:10 - 12:25 Short talk 6 selected from the abstracts
12:25 - 13:05 Mark Peifer (Chapel Hill): Building the body plan: the miracle of morphogenesis
13:05 - 14:05 Lunch

Session III

14:05 - 14:10 Introduction by the organizers
14:10 - 14:50 James Nelson (Stanford): Evolution of cell-cell adhesion and epithelial polarity
14:50 - 15:05 Short talk 7 selected from the abstracts
15:05 - 15:20 Short talk 8 selected from the abstracts
15:20 - 16:00 Rolf Kemler (Freiburg): β-Catenin and stemness
16:00 - 17:00 Poster Session / Coffee Break
17:00 - 17:40 Heiko Lickert (Neuherberg): Cilia and polarity shape the mouse embryo
17:40 - 17:55 Short talk 9 selected from the abstracts
17:55 - 18:10 Short talk 10 selected from the abstracts
18:10 - 18:25 Short talk 11 selected from the abstracts
18:25 - 19:05 Yohanns Bellaiche (Paris): Epithelial tissue morphogenesis: From multiscale imaging to local regulation of cell mechanical properties
20:00 Dinner at “Hotel Schwarzer Bär” Jena

Saturday, September 22

Session IV

08:55 - 09:00 Introduction by the organizers
09:00 - 09:40 Buzz Baum (London): A noisy path to order
09:40 - 10:20 Antonio Jacinto (Lisboa): Actomyosin pulses, flows and cables during epithelial repair
10:20 - 10:35 Short talk 12 selected from the abstracts
10:35 - 10:50 Short talk 13 selected from the abstracts
10:50 - 11:30 Coffee Break
11:30 - 11:45 Short talk 14 selected from the abstracts
11:45 - 12:25 Benedicte Sanson (Cambridge): In vivo mechanisms of collective cell movement and cell sorting
12:25 - 13:05 Jörg Großhans (Göttingen): Self-organisation of the syncytial nuclear array
13:05 - 13:20 Closing Remarks / Poster Award
13:30 Snacks and Departure
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SOLVING A BIG PUZZLE

Martin Beck

Multifunctional Gateways
In all eukaryotic cells chromatin is enclosed in the nuclear compartment. Nuclear pore complexes (NPCs) bridge the inner (INM) and outer nuclear membranes (ONM) and facilitate the nucleocytoplasmic exchange of molecules. NPCs tightly control nuclear protein transport and RNA export. They thus have an influence on both, the composition of the nuclear compartment and cytoplasmic processes involving RNA, e.g. translation and microRNA-based gene silencing. This function is realized by more than 20 soluble transport factors that recognize specific signal peptides, namely nuclear localization signals (NLS) and nuclear export signals (NES) of cargo molecules. Such transport factors guide cargos across the central channel of the NPC via as yet unknown mechanism (Wente and Rout, 2010). The nucleus of mammalian tissue culture cells contains more than 3000 different proteins and harbors about 2500 NPCs on its surface. Quantitative proteomics data indicate that each nucleus contains at least 568 protein molecules, half of which on average are turned over within 24 h (Beck et al., 2011; Boisvert et al., 2012). The sheer amount and complexity of distinct nuclear transport events that must occur in a coordinated fashion in life cells thus defies our imagination.

To provide the required capacity, the NPC as compared to other transporters, encompasses a rather large channel of ~50 nm in diameter (Beck et al., 2004). This is accomplished by 15 dedicated nucleoporins (in mammals) that are assembled in multiple copies into an eightfold rotationally symmetric scaffold that stabilizes the curvature of the joined INM and ONM. This scaffold is decorated with an additional 17 nucleoporins that are thought to provide functionalities specific to transport, namely docking sites for transport factors. Unlike the highly structured scaffold, these transport-functional Nups often contains intrinsically disordered domains and so-called Phenylalanine-Glycine (FG)-rich repeats.

Recent studies have described functions of Nups that go far beyond the classical view of their participation in nucleocytoplasmic exchange. As it turns out, Nups fulfill a variety of regulatory functions and are linked to cell cycle control, gene regulation, cell differentiation, a variety of human diseases and potentially even assembly of primary cilia (Capelson and Hetzer, 2009; Guttlinger et al., 2009; Kee et al., 2012).

Some of these functions take place in a variety of locations in the cell and are NPC-independent. Why the cell chooses to utilize the same proteins for building a membrane-bridging transporter and to carry out these other functions remains largely understudied. The picture is further complicated by the fact that NPCs themselves might be compositionally heterogeneous and devoted to specific transport and regulatory functions.

To understand the multi-functionality of Nups, a detailed structural map of the NPC is a crucial prerequisite. Thus far, the NPCs compositional complexity has prevented determination its structure at high resolution. This review discusses potential strategies towards tackling this challenge in the light of structural heterogeneity and dynamics of Nups.

Structural Analysis of Nucleoporins
The structure of a number of domain folds of scaffold Nups have been solved by X-ray crystallography in recent years (reviewed e.g. in (Bro-

Figure 1: Cryo electron microscopic map of the structure of the lower eukaryotic nuclear pore complex. (A) The scaffold structure of the Dichtystelium NPC is shov as isosurface-rendered view (at 8 Å resolution obtained by subtomogram averaging (Beck et al., 2007)). (B) The same as in (A) but displayed as cut-open view and segmented into membranes (gray), spoke (bright cyan), nuclear and cytoplasmic ring (dark cyan) structures.
hawn et al., 2009; Hoelz et al., 2011) and revealed insights into the structural organization on the lowest hierarchy level (highest resolution). The NPC scaffold structure consists primarily of two evolutionarily old structural motifs, namely α-only domain folds, called ACE1 (ancestral coatamer element one) and β-propellers (Brohawn et al., 2008). Currently ~26% of the nuclear pore is annotated with atomic models. Since both domain folds are highly repetitive elements, predictions can be made for a large fraction of the nuclear pore. Nevertheless, only a limited number of Nup interfaces have been solved, which is a considerable limitation for understanding NPC structure at its higher organization levels.

On the intermediate structural hierarchy level Nups are organized as hetero-oligomeric subcomplexes. During assembly of a NPC, multiple copies of these subcomplexes are joined together. As a consequence, subcomplexes are promising targets to obtain information on the intermediate structural level, which will be crucial to bridge the gap between the high and low resolution techniques. Currently, the best characterized module is the Y-shaped yeast Nup84 subcomplex (human homolog: Nups107). Several X-ray structures were assigned into a single particle EM map (Nagy et al., 2009) and a high resolution model of the entire subcomplex has been put forward (Fernandez-Martinez et al., 2012). Other subcomplexes have been defined biochemically, but structural information about them is sparse, with few exceptions (Amlacher et al., 2011; Solmaz et al., 2011). Precisely how subcomplexes assemble into NPCs remains a subject intense debate.

Three-dimensional electron microscopy (3D-EM) holds great potential to resolve this issue and has provided maps of the entire NPC that yielded insights into the structural organization on the highest hierarchy level (Grossman et al., 2012). However, the moderate resolution obtained thus far is not sufficient to annotate such maps with higher resolution structures. The attainable resolution is likely not limited by technological bottlenecks but rather the structural plasticity of NPCs per se, because flexible elements are ‘diluted out’ during the structure determination process. The development of algorithms, dedicated specifically to nuclear pores, that detect and account for such plasticity (Beck et al., 2007) is therefore necessary and might lead to further improvements in resolution in the future.

**Spatial and Compositional Complexity of Nuclear Pores**

A particular challenge everyone faces who works with nuclear pore complexes, is that they are very difficult to isolate because of their size, membrane-associated nature and intricate composition. Although NPC-enriched fractions were obtained from yeast and rat, they have been criticized for a potential loss of components (Hoelz et al., 2011). This problem is nicely illustrated by the history of the discovery of nucleoporins. In the early days, nuclear pores were assumed to be compositionally much more complex until systematic proteomic studies in yeast and rat demonstrated that the NPC is build from only about 30 different proteins (Cronshaw et al., 2002; Rout et al., 2000). Since then, classical cell biological approaches have occasionally led to the discovery of novel nucleoporins (see e.g. (Mansfeld et al., 2006; Rasala et al., 2006). There are a few factors that render the discovery of Nups challenging and might have prevented the generation of a complete compositional map until today: First, the mean residence times per NPC of some Nups is quite short (Rabut et al., 2004). Although this holds true only for a minority of Nups, it exposes the exciting possibility that there is a discovery bias towards long residing NPC members. Second, FG-rich domains often contain fewer cryptic sites, and many Nups are heavily post-transcriptionally modified. Both of these circumstances impose certain challenges for shotgun proteomic approaches. Lastly, Nups are evolutionarily relatively divergent and display a comparably low sequence conservation across species, although not as extreme as in the case of e.g. kinetochores (Meraldi, 2011).

Sequence alignments and more sophisticated bioinformatics approaches (Devos et al., 2004) have discovered a number of Nups in a variety of species but the relation of the functional homologies is often not clear. This is underlined by the fact that the phenotypes caused by systematic knock-out experiments in S. cerevisiae, S. pombe, A. nidulans, (Osmani et al., 2006) as well as gene silencing experiments in C. elegans and mammals are astonishingly inconsistent. The reason for these findings might be the modular organization of the NPC (Schwartz, 2005). The domain folds contained in Nups are highly redundant and possibly can compensate for losses of individual proteins.

Alternatively to working with entire NPCs, subcomplexes can be purified or reconstituted in vitro (Kampmann and Blobel, 2009; Sionov and Nissimov, 2000). Since nature utilizes subcomplexes as pre-assembled building blocks, protein interactions within subcomplexes are more stable while interfaces across subcomplexes are weaker and often regulated. As a consequence, our current understanding of the NPC is hampered by a discovery bias towards intra-subcomplex interactions, with very few exceptions (Mitchell et al., 2010; Sachdev et al., 2012). In order to satisfy the critical need for discovering more inter-subcomplex interactions newly emerging technologies have to be explored. These could for example be chemical modifications in combination with tandem mass spectrometry or high-mass electrospray approaches.

The general picture of NPC architecture discovered throughout species by cryoEM (Grossman et al., 2012), is that three ring-like structures, the cytoplasmic, spoke and nuclear rings, are stacked to stabilize the membrane curvature. Nuclear and cytoplasmic rings have larger diameters, while the smaller spoke ring is associated with the tip of the joined membranes (Figure 1). Taken together a much defined membrane curvature with a 30 nm diameter is formed (in mammals). Which Nups build up these structural elements remains largely elusive. Although the rough localization of Nups...
within the nuclear pore has been determined by immune-gold labeling and electron microscopy (Rout et al., 2000), the spatial accuracy of such experiments is not sufficient to precisely associate them with structural features evident by cryoEM. Since the protein folds of Nups are highly repetitive, fitting of high resolution structures is critically dependent on accurate positional information. Super-resolution microscopy techniques recently revealed the precise positioning of a few Nups, although only two-dimensionally within the nuclear envelope plane (see e.g., (Loschberger et al., 2012)). Alternatively, newly developed labels and markers for sub-three-dimensional electron microscopy might deliver the necessary information in the future. A striking feature of the cryo-EM reconstructions is that they show distinct, and as compared to the entire structure, relatively small membrane attachment sites. Interestingly, most Nups do not have the canonical properties of membrane proteins, except for three (NDC1, POM121 and gp210 in mammals) that harbor short transmembrane stretches. Instead, Nups seem to be similar to membrane coat proteins and might have maintained that mode of membrane association. The mammalian scaffold Nup133 possesses an amphipathic alpha-helical motif that specifically binds to membrane curvatures with small diameters (Drin et al., 2007). Similar motifs occur for example in the Golgi-associated protein ArfGap1. Two other membrane associated Nups, Sec23 and Sec7, are not exclusive members of the Nup but also integral parts of COPII coating complex and vacuoles (Dokudovskaya et al., 2011). Precise how the scaffold architecture is anchored to membranes remains elusive.

Cell-type specific Nuclear Pores?

Another layer of complication in understanding NPC architecture and function is its structural heterogeneity. It is in part caused by flexible elements but likely also by compositional variations that might occur on the single cell level but also across different cell types. The fact that NPCs are very long-lived and that the mean residence times per NPC of the majority of Nups is very long places the odds strongly in favor of context dependent rearrangements of NPC structure. Indeed, NPC remodeling has been reported in the context of HIV infection (Monette et al., 2011) and aging (D’Angelo et al., 2009) but also embryonic development (Asally et al., 2011; Feng et al., 2010; Lupu et al., 2008). Since the major function of the scaffold Nups is to stabilize the membrane gap, it is unlikely that the NPCs architecture is fundamentally remodeled as a function of the cell type. One might thus envision that the scaffold provides docking sites for a different subset of functional Nups that flavor the central channel in order to deal with the particularities of certain cell types, for example to activate different subsets of transport pathways or to foster interactions with different states of chromatin. The focus of future structural analysis might thus be to elucidate NPC architecture within context specific needs.

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- **Hervorragende Ergonomie**
- **Flexibel ausbaufähig**
Long non-coding RNA & the Hallmarks of Cancer

Tony Gutschner & Sven Diederichs

Abstract
Next generation transcriptome sequencing revealed that the human genome contains many more functional elements than just protein-coding genes. Up to 70% of the human genome is transcribed into RNA while only 2% are encoding for proteins. Thus, non-coding RNA is the major product of the human genome. In this review, we focus on the emerging role of long non-coding RNA (lncRNA) molecules in the field of tumor cell biology. Long lncRNAs are deregulated in many human cancer entities and show tissue-specific expression. Functional studies revealed a broad spectrum of mechanisms applied by lncRNAs such as HOTAIR, MALAT1, ANRIL or lincRNA-p21 to fulfill their functions at all levels of cell biology. Here, we link the cellular processes influenced by long lncRNAs to the hallmarks of cancer and provide an lncRNA perspective on tumor biology.

Introduction
Recent technological breakthroughs such as deep sequencing and tiling arrays revolutionized our view of genome organization and content as they revealed an unexpected finding: a much larger part of the human genome is pervasively transcribed into RNA than previously assumed. It is estimated that up to 70% of the genome is transcribed but only up to 2% of the human genome serve as blueprints for proteins. RNA molecules that lack protein-coding potential are collectively referred to as non-coding RNAs (ncRNAs). Well-known ncRNAs include classical "housekeeping" RNAs, such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), which are constitutively expressed and play critical roles in protein biosynthesis.

Long ncRNAs – What, Where and Why?
According to their size, ncRNAs are arbitrarily subdivided into two groups: small ncRNAs (<200 nt) and long ncRNAs. In recent years, small ncRNAs like microRNAs (miRNAs), small interfering RNAs (siRNAs) or PIWI-interacting RNAs (piRNAs) received most attention and especially miRNAs were shown to play many important roles in cancer. However, mammalian genomes encode also numerous long ncRNAs, defined as endogenous cellular RNAs of more than 200 nucleotides in length that lack an open reading frame of significant length (less than 100 amino acids). Therefore, long ncRNAs (lncRNAs) constitute a very heterogeneous group of RNA molecules that allows them to cover a broad spectrum of molecular and cellular functions by implementing different modes of action. LncRNAs often overlap with or are interspersed between coding and non-coding transcripts. Several recent studies identified thousands of lncRNAs in the human genome. For the vast majority of these recently discovered lncRNAs, the cellular function needs to be elucidated. Long lncRNAs are often expressed in a disease-, tissue- or developmental stage-specific manner and some are highly conserved throughout evolution pointing towards specific functions for these lncRNAs. Nevertheless, our knowledge of how lncRNAs can act in the cell and which roles they might play in diseases, e.g. cancer, is still very limited.

Only individual examples have been studied: LncRNAs play an important role in regulating gene expression at various levels, including chromatin modification, transcription and post-transcriptional processing. For example, the lncRNAs Xist (X inactive-specific transcript) or HOTAIR (HOX Antisense Intergenic RNA) interact with chromatin remodeling complexes to induce heterochromatin formation in specific genomic loci leading to reduced target gene expression. Long ncRNAs can also function by regulating transcription through a variety of mechanisms that include interaction with RNA binding proteins, acting as a co-activator of transcription factors, or repressing a major promoter of their target gene. In addition to chromatin modification and transcriptional regulation, long ncRNAs can modulate gene expression at the post-transcriptional level or splicing level. In Figure 1 we provide an overview about currently known lncRNA functions.

Hallmarks of Cancer – The basics
Cancer is one of the leading causes of death worldwide and accounted for 7.6 million (13% of all deaths) in 2008. Although "cancer" comprises a heterogeneous group of diseases, one characteristic and unifying feature is the creation of abnormal cells that grow beyond their natural boundaries. In

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2000, Hanahan and Weinberg proposed six hallmarks of cancer that all together form the fundamental principle of this malignant transformation³⁴. These basic hallmark capabilities of tumor cells are: (I) sustaining proliferative signaling; (II) evading growth suppressors; (III) enabling replicative immortality; (IV) activating invasion and metastasis; (V) inducing angiogenesis and (VI) resisting cell death. Over the last decade, remarkable progress was made in the field of cancer research which led to a better understanding of these hallmark capabilities, but also led to modifications and, ultimately, expansions of the original concept³⁵.

In this review, we further expand our thinking of the causes of cellular transformation by including long ncRNAs into cancer biology.

**Sustaining proliferative signaling**
One of the most prominent characteristics of a cancer cell is its ability to proliferate constantly and in the absence of external stimuli. How can ncRNAs affect this property? The **Steroid Receptor RNA activator (SRA)** is a coactivator for the steroid receptors for progesteron (PR), estrogen (ER), glucocorticoids (GR) and androgens (AR) increasing pro-proliferative signaling. SRA acts as an ncRNA³³ and is elevated in breast tumors³⁴. However, recent progress in this field has revealed a more complex situation: the SRA1 gene might not only act as an ncRNA but also produces a protein that acts as a coactivator or corepressor³⁶. Alternative splicing balances the ratio of non-coding and coding transcripts derived from the SRA1 gene³⁷. This duality of RNA transcripts with coding and non-coding functions adds another level of complexity to regulatory circuits in the cell. The well-known tumor suppressor p53 mRNA also exerts a novel, coding-independent function³⁸.

Other recently discovered long ncRNAs also play a role in cell proliferation. RNA-Seq technology identified 121 differentially expressed long ncRNAs in prostate cancer whose expression patterns distinguished benign, localized and metastatic prostate cancer³⁹. The IncRNA PCAT1 (prostate cancer associated transcript 1) modestly increased cell proliferation suggesting that PCAT1 might function as transcriptional repressor and might contribute to prostate cancer progression.

The small nuclear RNA 7SK, also known as RN7SK, regulates transcription elongation via binding to the positive transcription elongation factor b (P-TEFb) which abolishes its positive effect on RNA Polymerase II transcription elongation³⁴. HMGAI, a transcription factor and chromatin regulator, was now identified as a novel 7SK interaction partner³⁴. 7SK RNA interacts with HMGAI and competes with its binding to DNA affecting HMGAI target gene expression of growth-related genes.

**Evading growth suppressors**
A complementary cancer hallmark to sustaining proliferation is the evasion from growth suppressive signals of tumor suppressors such as TP53, PTEN or RB. In addition to well-known mechanisms of tumor suppressor inactivation such as mutation, deletion, epigenetic silencing or expression of inhibitors, cancer cells have developed alternative ways employing long ncRNAs.

**Five human ncRNA fragments interact with the tumor suppressor PSF³⁴. RNA bound to PSF releases it from the human proto-oncogene GAGE6 regulatory region resulting in an activation of GAGE6 expression. A different mode of action is executed by the long ncRNA ANRIL (antisense non-coding RNA in the INK4 locus) to block the activity of tumor suppressor genes³⁴. ANRIL interacts with SUZ12 (suppressor of zeste 12 homolog), a subunit of the polycomb repression complex 2 (PRC2) and recruits the complex to repress the expression of the cell cycle inhibitor p15 (INK4B), a well-known tumor suppressor gene. A recent study identified >9000 PRC2-interacting RNAs via RIP-Seq (RNA immunoprecipitation and sequencing) in embryonic stem cells making it very likely that many more genes are regulated by the ncRNA-mediated recruitment of PRC2³⁵.

In contrast to these oncogenic ncRNAs, the ncRNA GAS5 (Growth Arrest-Specific 5) is a tumor suppressor and contributes to growth suppression. It was identified based on its increased levels in growth-arrested mouse NIH3T3 fibroblasts³⁶. It contains a small and poorly conserved open reading frame that does not encode a functional protein³⁴. GAS5 is the host gene for multiple snoRNAs, which are located in the introns and may mediate important biological activities³⁶. GAS5 functions as a “riborepressor”⁴⁰: the ncRNA interacts with the DNA binding domain of the glucocorticoid receptors, thus competing with the glucocorticoid response elements in the genome for binding to these receptors. This suppresses the induction of several responsive genes including cellular inhibitor of apoptosis 2 (cIAP2) and ultimately sensitizes cells to apoptosis³⁶. Another growth-suppressive ncRNA was found by asking how the transcription factor TP53 can do both, activate and repress gene expression³⁴. The **lincRNA-p21** is a direct p53 target gene residing next to the p21 (Cd-kn1a) gene on mouse chromosome 17. Its expression is activated upon DNA damage and it associates with hRNP K, a well-known RNA binding protein and transcriptional repressor. LincRNA-p21 mediates the binding of hRNP K to its target genes, which finally leads to gene silencing and the induction of apoptosis.

Interestingly, the expression of the tumor suppressor p21 is regulated by at least one other non-coding transcript: a **p21-specific antisense transcript**, which functions in Argonaute 1-mediated transcriptional control of p21 mRNA expression³⁴.
Enabling replicative immortality
Linked to the first two hallmarks is the third trait of cancer: unlimited replicative potential. Normal cells are able to pass through only a limited number of cell division cycles. In contrast, tumor cells show nearly an unlimited replication span. The chromosome ends, the telomeres, are crucial for this replication limit as they shorten after each cell division. Tumor cells have found two ways to circumvent the loss of telomeres: (I) About 90% of all human cancers express a specialized enzyme, called telomerase, which is able to add telomeric repeats to the end of the chromosomes. (II) The remaining 10% of all tumor cells employ alternative lengthening of telomeres (ALT), a non-conservative telomere lengthening pathway involving the transfer of telomere tandem repeats between sister chromatids.

The major pathway involving telomerase critically depends on an ncRNA. The telomerase holoenzyme consists of a protein component, a reverse transcriptase named TERT (Telomerase Reverse Transcriptase) and an RNA primer, also known as TERC (Telomerase RNA Component) or TR (Telomerase RNA)². The telomeric RNAs are highly divergent between different species, varying in both size and sequence composition, from ~150 nt in ciliates and ~450 nt in vertebrates up to ~930-1300 nt in the budding yeast. For a more detailed discussion on TERC structure and function please refer to Theimer and Feigon³.

Another group of long ncRNAs named TERRA (telomeric repeat-containing RNA)⁴⁻⁵ are derived from subtelomeric loci. TERRA localizes to telomeres and is involved in telomeric heterochromatin formation⁶. TERRA is thought to be a global or local negative regulator of the telomerase enzyme⁷. TERRA forms a complex with the proteins hnRNP A1 and POT1 (protection of telomeres 1) promoting telomere capping and preserving genomic integrity⁸. TERRA reduction is required for telomerase-mediated telomere lengthening which may link TERRA to cancer. However, a deregulation, silencing or mutation of TERRA in human cancer remains to be discovered.

Activating invasion and metastasis
The fourth hallmark capability is the ability of tumor cells to invade into neighboring tissues and form distant metastases. The first lncRNA associated with metastasis is MALAT1, the Metastasis-Associated Lung Adenocarcinoma Transcript 1, that was initially published as MALAT1 and later also referred to as NEAT2 (Nuclear-Enriched Abundant Transcript 2). It was discovered as a prognostic marker for metastasis and patient survival in early stages of non-small cell lung cancer (NSCLC)⁹. This ncRNA is extremely abundant in many human cell types and is highly conserved across several species underscoring its functional importance¹⁰. Additionally, the 8 kb long MALAT1 can be processed into a highly conserved tRNA-like small cytoplasmic RNA of 61 nucleotides that is broadly expressed in human tissues¹¹. However, the function of this so-called masRNA is so far unknown. MALAT1 is retained in the nucleus and specifically localizes to nuclear speckles¹² - structures that play a role in pre-mRNA processing. MALAT1 might regulate alternative splicing of pre-mRNA by modulating the levels of active serine / arginine splicing factors¹³. Depletion of MALAT1 alters the processing of a subset of pre-mRNAs, which play important roles in cancer biology, e.g. Tissue Factor or Endoglin¹⁴. However, a recent study indicates additional functions for MALAT1 in the nucleus¹⁵. MALAT1 interacts with the unmethylated form of CBX4/PC2 in the Polycomb Repressive Complex 1 (PRC1). This interaction controls relocation of growth control genes between polycomb bodies and interchromatin granules, places of silent or active gene expression, respectively. Therefore, the exact mechanism of MALAT1 function is still a mystery. MALAT1 might function cell type- or tissue-specific despite its ubiquitous expression pattern. MALAT1 expression can be found in many healthy organs with the highest level of expression in pancreas and lung⁶. In several human cancers including lung cancer, uterine endometrial stromal sarcoma, cervical cancer and hepatocellular carcinoma (HCC), MALAT1 is upregulated⁶,⁶⁰,⁶¹. In addition, it is significantly associated with metastasis and can serve as an independent prognostic parameter for patient survival in early stage lung adenocarcinoma⁶⁰,⁶¹. MALAT1 promotes cell motility of lung cancer cells through transcriptional or post-transcriptional regulation of motility-related genes⁶². Additionally, MALAT1 supports proliferation and invasion of cervical cancer cells and regulates caspase-8, caspase-3, Bax, Bcl-2 and Bcl-xL⁶³. Thus, MALAT1 is linked to a plethora of potential functions in proliferation, apoptosis, migration or gene regulation and future studies will have to unravel the specificity of these effects. Since multiple studies were carried out with individual siRNAs only, further investigations are necessary to ensure the specificity of the observed effects and to corroborate the functional importance of MALAT1 in carcinogenesis or metastasis. For this reason, we have recently developed a novel gene knockout strategy based on the stable bi-allelic integration of RNA destabilizing elements into the human genome with the help of Zinc Finger Nuclease⁶⁴. This method yields a highly specific and more than 1000-fold reduction of MALAT1 expression in human A549 lung cancer cells and will allow the first quantitative loss-of-function analysis of MALAT1 function in lung cancer and its role in migration and metastasis. A second long ncRNA involved in metastasis is HOTAIR (HOX Antisense Intergenic RNA). HOTAIR is a 2.2 kb long ncRNA transcribed in antisense direction from the HOXC gene cluster⁶⁵. HOTAIR functions in trans by interacting and recruiting PRC2 to the HOXD locus which leads to transcriptional silenci-
ng across 40 kb. HOTAIR also interacts with a second histone modification complex, the LSD1/COREST/REST complex, which coordinates targeting of PRC2 and LSD1 to chromatin for coupled histone H3K27 methylation and K4 demethylation\(^5\). Given its important role in the epigenetic regulation of gene expression, it is not surprising that HOTAIR is deregulated in different types of cancer\(^5\). In human breast cancer, HOTAIR expression is increased in primary tumors and metastases and its expression level in primary tumors positively correlates with metastasis and poor outcome. Overexpression of HOTAIR in epithelial cancer cells alters H3K27 methylation via PRC2 leading to increased cancer invasiveness\(^5\). In HCC, HOTAIR levels are increased compared to non-cancerous tissues and for those HCC patients, who received a liver transplantation, high HOTAIR expression levels are an independent prognostic marker for HCC recurrence and shorter survival\(^5\). Similar to breast cancer, HOTAIR depletion in liver cancer cells reduces cell invasion and cell viability\(^5\).

**Inducing angiogenesis**

When tumor mass and size increases, it is limited by the natural diffusion limit of oxygen and nutrients until tumor cells acquire the fifth trait: the ability to induce angiogenesis. The natural antisense transcript (NAT) aHIF is complementary to the 3’ untranslated region of the hypoxia inducible factor alpha (HIF-1α) and negatively regulates the expression of HIF-1α, a critical regulator of angiogenesis constituting a negative feedback loop\(^5,7\). aHIF is a marker for poor prognosis in breast cancer\(^5\).

Another NAT linked to angiogenesis is part of the transcriptional unit of the human endothelial nitric-oxide synthase, eNOS. The transcript was termed sONE or NOS5AS and regulates the expression of eNOS in a post-transcriptional manner under normoxia and hypoxic conditions\(^5\). At the moment, it is a matter of debate, whether sONE acts as an RNA, because also a protein product has been described\(^5\).

Finally, a NAT for tyrosine kinase containing immunoglobulin and epidermal growth factor homology domain-a (tie-4L)\(^9\) has been identified: tie-4AS.\(^8\) This long ncRNA is conserved in zebrafish, mouse and humans where it selectively binds to tie-1 mRNA in vivo and regulates tie-1 transcript levels, resulting in specific defects in endothelial cell contact junctions. In addition, the ratio of tie-1 versus tie-4AS is altered in human vascular-related disease states.

**Resisting cell death**

Immortality – for cancer cells it can become true, if they acquire the last hallmark capability: resisting cell death. **PCGEM1** (Prostate-specific transcript 1) is a prostate-specific and cancer-associated long ncRNA.\(^2\) It functions in apoptosis inhibition after doxorubicin treatment of prostate cancer cells.\(^2,8\) The anti-apoptotic effect is androgen-dependent and might result from a delayed induction of p53 and p21(WAF1/CIP1) after PCGEM1 overexpression.

A global approach using differential display identified genes conveying drug resistance

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**Figure 1: Cellular functions of long ncRNAs**. LncRNAs can act in diverse ways in the cell. Regulating gene expression is one of the best studied functions of lncRNA and multiple mechanisms are applied by lncRNAs: A) lncRNAs could be processed into small, single- or double-stranded RNAs that could act as endo-siRNAs targeting other RNAs. B) LncRNAs can act as “miRNA sponges” and sequester miRNAs to inactivate these small regulatory RNAs. C) The interaction of lncRNAs with proteins can modulate protein activity and localization. For example, the lncRNA NRON (non-coding repressor of Nfat) binds to the cellular transcription factor Nfat (nuclear factor of activated T-cells) regulating nuclear-cyttoplasmic trafficking of Nfat. D) LncRNAs regulate gene transcription via recruiting transcription factors to their target gene promoters, therefore activating gene expression. Vice versa, they can also block binding of general transcription factors, potentially via formation of RNA-DNA-Complexes. E) LncRNAs contribute to transcriptome complexity, as they can regulate alternative splicing of pre-mRNAs. G) The balance between transcriptionally active euchromatin and silent heterochromatin is controlled by lncRNAs. They can interact with chromatin remodeling complexes and induce local or global changes in chromatin packaging. Furthermore, lncRNAs can influence protein localization (D) and are important for the formation of cellular substructures or protein complexes, where they fulfill scaffolding functions (C, H).
to cancer cells and led to the discovery of **CUDR** (cancer up-regulated drug resistant)\(^5\). CUDR confers resistance to doxorubicin and etoposide as well as drug-induced apoptosis in squamous carcinoma cells - potentially via the downregulation of effector caspase 3.

More long ncRNAs could play a role in cell death control and might be evolutionarily conserved\(^6\). This study identified several hundred transcripts derived from ultra-conserved regions, **T-UCRs**, which are consistently altered at the genomic level in human leukemias and carcinomas. The depletion of the ncRNA uc.73A(P), a T-UCR significantly upregulated in colon cancer, resulted in reduced cellular proliferation of COLO-205 cells and an increase in sub-G1 cells, suggesting higher apoptosis rates.

Several long ncRNA are differentially expressed in melanoma cell lines in comparison to melanocytes and keratinocytes including **SPRY4-IT1** which is derived from an intron of the **SPRY4** gene\(^7\). SPRY4-IT1 is predominantly localized in the cytoplasm of melanoma cells, and its knockdown results in defects in cell growth, differentiation, and higher rates of apoptosis in melanoma cell lines.

Finally, DNA damage can induce five long ncRNAs from the p53 promoter including **PANDA** (P21 associated ncRNA DNA damage activated)\(^8\). PANDA acts in trans via interaction with the transcription factor NF-YA and limits the expression of pro-apoptotic genes. Consequently, PANDA depletion markedly sensitized human fibroblasts to apoptosis by doxorubicin.

DNA damage also leads to the activation of **cis-acting ncRNAs** derived from regulatory regions of the human **CCND1** promoter\(^9\). These ncRNAs bind to the TLS protein (translocated in liposarcoma), which inhibits cyclin D1 expression via interaction with and inhibition of CBP (CREB-binding protein) and p300.

### Long ncRNAs – Future challenges

Taken together, an overwhelming amount of data strongly emphasizes the functional importance of long ncRNAs in all fields of cell biology and provide first mechanistic insights how long ncRNAs can contribute to the hallmark capacities of cancer cells. A general list of IncRNAs with a connection to cancer has also been recently established\(^10\).

Long ncRNAs have proven to be important regulators in health and disease. However, only individual examples have been functionally studied in detail so far and many important questions remain to be addressed:

1. **Meticulous definition of the sequence identity of IncRNAs in normal and cancer cells**;
2. **Functional characterization of IncRNAs at the cellular level**;
3. **Determination of the molecular functions and relevance of IncRNAs in physiological and pathological settings**;
4. **Understanding the complex interplay between IncRNAs and proteins in the cell in health and disease**.

Novel techniques will foster future research breakthroughs in this field, such as screening methods for RNA bound to chromatin (ChIRP or CHART\(^11\)) or bound to proteins (RIP-Seq or PAR-CLIP\(^12\)).

Long ncRNAs specifically expressed or silenced in human cancers could play an important role in these cancer entities and therefore might represent novel therapeutic target genes or valuable biomarkers.

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Astrocyte secreted Sonic Hedgehog regulates BBB properties and endothelial immunequiescence.

Jorge Ivan Alvarez

The blood-brain barrier (BBB) confers homeostasis to the central nervous system (CNS) and limits entry of blood-borne molecules and circulating immune cells into the brain and spinal cord. The BBB is composed of specialized endothelial cells (ECs) held together by multiprotein complexes known as junctional proteins\(^4\). Astrocytes, which are in close apposition to the cerebral vasculature, help maintain BBB integrity and immune quiescence through contact dependent mechanisms and through release of soluble factors\(^4\). In the course of neuro-inflammatory conditions, such as multiple sclerosis (MS), a breakdown in the BBB occurs, allowing leukocytes to enter the brain parenchyma leading to demyelination and neuronal damage\(^8\). As astrocytes are key regulators of BBB properties, the understanding of astrocyte-EC interactions at the level of the neurovascular unit (NVU) may lead to the identification of novel molecular pathways and strategies to dampen CNS inflammation, or conversely to facilitate drug entry into the CNS.

The Hedgehog (Hh) pathway is a conserved signaling cascade involved in embryonic morphogenesis, neuronal guidance and angiogenesis\(^2\). In adult tissues, it plays an important role in vascular proliferation, differentiation and tissue repair\(^2\). Hh signaling is initiated by one of three secreted homologues of the Drosophila Hh: Indian Hh (Ihh), Desert Hh (Dhh) and Sonic Hh (Shh), the latter being widely associated with CNS morphogenetic events\(^3\). Essential to Hh signaling is the internal autocatalytic cleavage of the 45 kDa protein to yield the ~19 kDa N-terminal domain. Shh is released from the producing cell and binds with high affinity to the cell surface receptor Patched (Ptc)-1. Engagement of Ptc-1 alleviates repression of the signal transducer Smoothened (Smo), which then activates the transcription factors of the glia-associated antigen (Gli) oncogene family\(^8\).

Studies on the involvement of Shh in MS and its animal model, experimental allergic encephalomyelitis (EAE), have been mostly focused on the role of Shh in oligodendrocyte maturation\(^6\), a crucial step in new myelin formation. A recent study has also demonstrated Shh expression in astrocytes within demyelinated and remyelinating lesions in EAE\(^9\). In the peripheral nervous system, Dhh signal is required for the formation and maintenance of the perineurium\(^10\). By analogy, our current study explores whether the Hh pathway plays a role in CNS barrier formation and maintenance, such as the BBB. To determine whether components of the Hh pathway are expressed by the BBB, mRNA and protein analyses were performed using primary cultures of human fetal astrocytes (HFAs) and human adult BBB-ECs. HFAs expressed Shh mRNA, while BBB-ECs did not (Fig. 1a). In contrast, low levels of receptor Ptc-1 and Smo mRNA expression were observed in HFAs, and higher levels were found in BBB-ECs (Fig. 1a). Protein analyses revealed that whole cell lysates derived from HFAs contained the 45 kDa uncleaved precursor form of Shh protein (Fig. 1b). While BBB-ECs did not produce Shh and Ptc-1 expression on these cells was confirmed at the protein level (Fig. 1b). Immunocytofluorescence demonstrated intracellular localization of Shh in HFAs (Fig. 1c upper panels) which was not found in BBB-ECs (not shown). Conversely, membrane-bound Ptc-1 and Smo were detected in cultured BBB-ECs (Fig. 1c – medium and lower panels), but not on the surface of astrocytes. The expression of these Hh components was confirmed in situ using control human adult brain sections (data not shown). Therefore, our data show that human astrocytes express and secrete Shh. Brain endothelium, on the other hand, expresses the Hh receptors Ptc-1 and Smo, supporting the possibility that the Hh pathway is used for the communication between perivascular astrocytes and BBB-ECs. Primary cultures of confluent human BBB-EC monolayers form a semi-permeable barrier that, in presence of astrocyte conditioned media (ACM), further restricts the diffusion of soluble tracers\(^10\). The influence of the Hh pathway in the barrier properties of these cells was established by analyzing their transendothelial electric resistance (TEER) in conditions stimulating and abrogating the Hh pathway. Activation of the pathway using human recombinant Shh (hrShh; 0.1 μg/ml) and the Smo non-peptide receptor agonist purmorphamine (1 μM) induced a significant increase in the TEER of primary BBB-ECs that is comparable to the effect induced by 40% ACM (v/v) (Fig 1d). In contrast, treatment with the antagonist Cyclopamine reversed this effect (Fig 1d). To assess whether activation of the Hh pathway was responsible for the barrier promoting effects of ACM, the permeability of BBB-ECs in the conditions previously described was established. Purmorphamine and hrShh reproduced the effect of ACM in restricting the permeability to fluorescein isothiocyanate-labeled bovine
Figure 1: The expression of Hh components at the level of the neurovascular unit induces BBB properties. (a) RT-PCR of Shh, Patched-1 (Ptc-1) and Smoothened (Smo) in primary cultures of human fetal astrocytes (HFS; two preparations: lanes 1 and 2) and human adult blood-brain barrier endothelial cells (BBB-ECs; two preparations: lanes 3 and 4). (b) Western blot (WB) of Hedgehog (Hh) pathway components in BBB-ECs and HFS. WB of Ptc-1 (150 kDa) and Shh (42 kDa) in BBB-ECs and in precipitated astrocyte-conditioned media (ACM). (c) Protein localization of Hh pathway components in HFS and BBB-ECs grown in culture. Intracellular staining of Shh (green) in GFA immunopositive HFS (red). Surface staining of Ptc-1 (red), and the co-receptor Smo (red) in BBB-ECs. Merged images are shown (right panels). Hoechst-stained nuclei are blue. Scale bars, 50 μm. Transendothelial electric resistance (d) and permeability to 14C Sucrose and BSA-FITC (e) of human BBB-EC monolayers stimulated for 24 h with astrocyte-conditioned media (ACM) 40% V/V, hrShh (0.1 μg/ml), ACM plus Cilastatin (30 μM) or Purmorphamine (1 μM). (f) Quantitative real-time PCR of Ptc-1, claudin-5 and occludin mRNA extracted from BBB-ECs grown with ACM or with hrShh for 24 h (0.1 μg/ml). (g) WB analysis of claudin-5, occludin, JAM-A, claudin-3 and VE-cadherin in protein lysates from BBB-ECs after treatment with ACM or ACM + cyclophilin for 24 h. (h) CNS sections of 8 w old WT (upper panels) and Tie2-Cre; Smofl (lower panels) animals injected intra-peritoneally with Evans blue-EB (red) and immunostained for laminin-lam (green and labeling basement membrane of blood vessels) and IgGs (blue). White rectangles indicate areas enhanced on the right panels. Arrowheads denote disrupted/lower laminin expression. (i) CNS sections of 8 week old WT (upper panels) and Tie2-Cre; Smofl (lower panels) animals injected intra-peritoneally with Evans blue (red) and immunostained for PECAM-1 (green). Panels on the right represent the pixel intensity for the molecules in the path of the selected area (white line) and insets on the top right are images of the brains after sacrifice. (j) WB analysis of the junctional proteins claudin-5, occludin, p120 and ZO-1 extracted from CNS microvessels isolated from 8 wk old WT and Tie2-Cre; Smofl animals. (k) Characterization of CNS vessels in P19 WT and Tie2-Cre; Smofl mice. Immunofluorescence for GFAP (astrocytes), PECAM-1, claudin-5, claudin-3, occludin, ZO1, p120 (all BBB endothelial markers), fibrinogen and immunoglobulins G (IgGs, indicators of plasma protein leakage). White rectangles indicate areas shown on the right of each panel. Arrowheads on occludin panels indicate vessels. Nuclei (TOPRO-3, blue). Scale bars: 30 μm. Error bars, mean ± SEM. *, P<0.05; **, P<0.01. Reproduced with permission from Alvarez et al., 2011.
serum albumin (FITC-BSA) and "C-Sucrose (Fig. 1e), while cycloamine reversed the barrier promoting effects of ACM, resulting in an increase of permeability that compared to the levels of untreated BBB-ECs (Fig. 1e). These experiments confirm that Smo engagement influences the paracellular diffusion of small and large molecular weight tracers, possibly through tight junction (TJ) protein regulation. Altogether, our data demonstrate that astrocyte-produced Shh promotes barrier properties in human BBB-ECs through activation of Smo.

As TJs are the anatomical basis of the barrier properties in the CNS vasculature, the influence of the astrocyte-derived Shh signal in the expression of these proteins was determined in human BBB-ECs. BBB-ECs grown with ACM or hrShh for 24 h upregulated Ptc-h1 mRNA expression (Fig. 1f) confirming Hh pathway activation in either case. In addition, mRNA of the TJ proteins occludin and claudin-5 were also upregulated in BBB-ECs cultured for 24 h with ACM or hrShh (Fig. 1f). Western blot analyses revealed a moderate increase in the expression of claudin-5, occludin, junctional adhesion molecule (JAM)-A, claudin-3 and VE-cadherin when BBB-ECs were treated with ACM (Fig. 1g). However, a significant decrease in expression of these TJ molecules was observed when Smo was antagonized by cycloamine treatment (Fig. 1g). Taken together, our data demonstrate that Shh is important in TJ maintenance in human adult brain ECs.

To specifically assess the role of the Hh pathway at the BBB level, we selectively deleted a conditional allele of the signal transducer Smo from ECs using a Tie2-Cre recombination driver (Tie2-Cre; Smofl/fl). The specific deletion of smo in ECs was associated with a significant increase in the permeability of the BBB to endogenous and exogenous permeability tracers at E14, E18, P4, P19 and during adulthood (Figs. 1h and 1i). The plasma protein leakage into the CNS correlated with a decrease in the expression of the junctional proteins claudin-5, occludin, ZO-1 and p120 (Figs. 1j and 1k). Moreover, in the Tie2-Cre; Smofl/fl mice the basement membrane casing of blood vessels was compromised as breaking of the extracellular matrix component laminin was detected in the periphery of parenchymal vessels (Fig. 1h), while the association of astrocyte endfeet at the level of the glia limitans was considerably reduced (Fig. 1k). Thus, the genetic neutralization of the Hh pathway in CNS endothelium resulted in lower expression of vascular junctional proteins and accumulation of blood products in the CNS, hallmarks of a perturbed and compromised BBB.

Under normal conditions, the restrictiveness of the CNS to immune responses is in part attributable to the BBB. To determine the role played by Shh in this process, the production of leukocyte chemoattractants by BBB-ECs was studied under conditions stimulating and blocking the Hh pathway. Upon treatment of human brain ECs with hrShh or purmorphamine, the secretion of chemokines IL-8/CXCL8 and monocyte chemoattractant protein 1 (MCP-1)/CCL2 was significantly reduced (Fig. 2a), while Smo inactivation with cycloamine abrogated this effect (Fig. 2a). The role of Shh in the expression of cell adhesion molecules (CAMs) on the surface of BBB-ECs was also studied. Under basal conditions, primary cultures of BBB-ECs express intercellular adhesion molecule (ICAM)-1 (Fig. 2b). When grown with 40% ACM (v/v), the percentage of cells expressing ICAM-1 was significantly reduced. A similar decrease was seen in BBB-ECs cultured in the presence of hrShh or the Hh pathway agonist purmorphamine (Fig 2b). On the other hand, blockade of the Hh pathway with cycloamine reverted ICAM-1 expression to basal levels.

Given the role of chemokines and CAMs in cell adhesion and diapedesis, the migration of CD4+ T lymphocytes across Hh-activated BBB-ECs was assessed. Transmigration of unstimulated human CD4+ T cells through BBB-ECs was significantly reduced when BBB-ECs were cultured with ACM, hrShh or purmorphamine (Fig. 2c) and significantly increased with Cycloamine treatment (Fig. 2c). To further characterize the role of the Hh pathway in inducing immune quiescence of the CNS compartment, the adhesiveness of T helper (Th) cells to BBB-ECs and the phenotype of human Th lymphocytes skewed under Hh growing conditions were studied. CD14+ monocytes and CD4+ T cells expressed Ptc-h1 and Smo as well as the Hh transcription factor Gli-1 (data not shown), indicating that these cells can respond to Shh. As T cell binding to BBB-ECs is the first step in their transmigration to the CNS, we determined the effect of Shh in the adhesiveness of human Th17, Th1 and Th2 lymphocytes to BBB-ECs. The adhesion of Th17 and Th1 cells, which are suspected to participate in neuroinflammatory processes like MS, to human BBB-ECs was significantly reduced when Th lymphocytes were polarized and expanded in the presence of Shh (Fig 2d). Adversely, adhesion of Th2 cells to BBB-ECs was significantly enhanced (Fig. 2d). To elucidate the effect of Shh on the adhesion capacity and activation of Th cells, we studied the expression of molecules known to participate in leukocyte migration through the BBB in MS. Thus, expression of CD6, lymphocyte function-associated antigen (LFA)-1 and very late antigen (VLA)-4 as well as the cytokines interferon (IFN)-γ and interleukin (IL)-17 were determined on Th1, Th17 and Th2 cells. Shh decreased the expression of CD6 on Th17 cells (Figs. 2e and 2h), while the levels of LFA-1, VLA-4 and IL-17 remained similar to the control (data not shown). In contrast, an increase in the expression of CD6 and VLA-4 was seen on Th2 cells skewed in the presence of Shh (Figs. 2f and 2h). In Th1 cells, Shh reduced IFN-γ production (Figs. 2g and 2h), but did not alter the expression of CD6, LFA-1 and VLA-4 (data not shown). We did not detect any effect of Shh on the expression or secretion of IL-
Figure 2. The Hh pathway promotes immunoenquiescence of the brain endothelium and dampens neuroinflammatory responses. (a) Chemokine secretion by BBB-ECs treated for 24 h with hrShh, purmorphamine or both cyclopamine and ACM. IL-8/CXCL8 and MCP-1/CCL2 secretion by BBB-ECs was detected by ELISA. (b) Flow cytometric analysis of ICAM-1 expression by BBB-ECs treated with ACM, ACM + cyclopamine, hrShh or purmorphamine for 24 hrs. (c) CD4+ T lymphocyte migration was evaluated using the modified Boyden chamber assay. 2×10^5 human CD4+ lymphocytes were allowed to migrate across BBB-ECs under conditions stimulating or antagonizing the Hh pathway for 24 h. (d) Human CD4+CD45RO+ T lymphocytes were polarized and expanded into Th17, Th1 and Th2 cells in the presence or absence of hrShh (0.1 μg/ml) and were allowed to adhere for 2 h to untreated BBB-ECs. (e) Flow cytometry analysis of CD6 expression in Th17 cells expanded in the presence (blue) or absence (red) of hrShh (0.1 μg/ml). Black histogram represents isotype control. (f) Flow cytometry analysis of CD6 (left panel) and VLA-4 (right panel) expression in Th2 cells differentiated in the presence (blue) or absence (red) of hrShh (0.1 μg/ml). (g) Flow cytometry density plot of intracellular IFN-γ and IL-17 expression by Th1 cells in the presence (right panel) or absence (left panel) of hrShh (0.1 μg/ml). (h) Total percentage of representative histograms shown in figs 2e to 2g. (i to m) Outcome of Hh neutralization using the Smo antagonist GDC-0449 (GDC; 25 mg/Kg, injected at days 0, 4, 8 and 12, arrowheads) on the clinical score of EAE mice (i), on their weight change (j), on the extent of spinal cord demyelination (arrowheads indicate border of demyelinating lesions) (k), on the number of immune cells accumulating into the CNS (l), and on the expression of IFN-γ and IL-17 by CD4+ T cells infiltrating the CNS (m), as compared to vehicle (DMSO). Error bars, mean ± SEM. *P<0.05; **P<0.01; ***P<0.001. Scale bars: 50 μm. Reproduced with permission from Alvarez et al., 2011.

17 by Th17-polarized lymphocytes (data not shown). These findings correlate with the pattern of adhesiveness shown in Fig 2d and collectively support an endogenous immune quiescent role of the Hh pathway at the level of the BBB, protecting the CNS against entry of pro-inflammatory lymphocytes.

As Hh activating conditions have immunomodulatory effects on BBB components, the influence of Hh pathway was then assessed in EAE, the animal model of MS, by repeated injections of the Hh antagonist GDC-0449. Hh antagonism significantly increased disease severity as clinical scores and weight loss were significantly higher in mice treated with GDC-0449 (Figs. 2i and 2j). Blocking of the Hh pathway also increased the extent of demyelination (Fig. 2k), the amount of leukocytes accumulating in the CNS (Fig. 2l), and the production of IFN-γ and IL-17 in T cells infiltrating the CNS (Fig. 2m). The magnitude of T cell activation in the GDC-0449 treated animals was unique to the CNS compartment as the degree of T cell activation in the periphery did not differ between both groups (data not shown). Collectively, these data support an endogenous immune quiescent role of the Hh pathway at the level of the BBB by protecting the CNS against entry of pro-inflammatory lymphocytes. The ensheathing of CNS microvessels by astrocyte endfeet is known to provide soluble factors needed for optimal development and maintenance of an impermeable BBB in vitro. Morphogens are believed to participate in astrocyte-endothelial interactions, as proteins of the fibroblast growth factor (FGF) and Wnt families are known to promote junctional protein expression, TJ strand formati-
on and impermeability of the BBB. The expression of Shh by human and mouse astrocytes have been previously described, however Shh function, especially in the context of BBB integrity and CNS immunity, remained unexplored. Shh has been involved in a multitude of developmental processes, ranging from embryonic tissue patterning to capillary morphogenesis. Evidence of the Hh pathway involvement in epithelial barrier formation has been shown in the developing submandibular gland, where lumen formation and TJ assembly is a process dependent on Shh. Additionally, in the peripheral nervous system, Dhh expressed in myelinating Schwann cells, partakes in the formation and maintenance of the perineurium, a structure reminiscent of the BBB. Therefore, our data further demonstrate the potential of the Hh pathway in the formation of a mature, ordered and functionally competent CNS barrier, as Hh neutralization reduced the expression of junctional proteins in primary cultures of human BBB-ECs and in the CNS of Tie2-Cre; Smo+/- mice.

The BBB is an important entity in keeping the CNS in a relative immunological privilege condition. Evidence presented here suggests a novel and unique role for Shh in regulation of CNS immunity. BBB-ECS treated with hShh or with Smo agonist purmorphamine, significantly reduce their secretion of chemokines, their expression of CAMs and their ability to support transmigration of CD4+ T cells. In addition, polarization of T helper lymphocytes in the presence of Shh resulted in reduced adhesion of inflammatory Th17 and Th1 cells to BBB-ECS, while adhesion of Th2 cells was enhanced. Such phenotype was associated with changes in the expression of immune mediators known to be important in the pathogenesis of MS and EAE such as IFN-γ, CD6 and VLA-4, and demonstrates that Shh acts as an endogenous anti-inflammatory effector of the neurovascular unit.

As a whole, the data presented here indica-

tes that the Hh pathway plays a dual protective role at the level of the BBB. Hh activation during embryonic development and adulthood promotes TJ expression and barrier formation, but also acts as an endogenous anti-inflammatory signal able to downregulate immune activation of the brain endothelium and thus impact on the recruitment of inflammatory T cells. In addition, these novel findings open a new frontier in the field of therapeutics targeting leukocyte migration into the CNS or conversely, to augment delivery of chemotherapeutic agents in the brain.

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Expand Knowledge – Change Ideas – Discover Hot Topics – Shape Science

Harald Herrmann

This motto has been propagated by the British Biochemical Society on the occasion of its “100 years” celebration, and it was used as a page heading within a (paper) notebook that I have received last year at a meeting of the Biochemical Society in Cambridge. On the inside page of the notebook, the society officials had “advertised” a mission statement that I found interesting enough to have it repeated - and commented on – here. Their mottoes also illustrate my motivation to continue, after I have stepped down as the president of our society, as an “editor-in-chief” of Cell News. Although we have a board of editors, it appeared at some point within the last two years that we need someone who cares about the timing of the publication, if we indeed do intend to have the journal published regularly.

And here they are - “Supporting the molecular bioscience community”: (I) to reveal “hot topics” at scientific conferences; (II) to “influence policy by approaching the public”; (III) to promote bioscience by engaging members of the society in “education and learning”; (IV) to provide funding in order “to support career development”; (V) to publish “high quality journals”. Points one to four represent exactly what the DGZ tries to provide, and how do we realize such aims? This year, members of the DGZ organized five meetings, four of them still to come: The annual meeting with an exceptional program, the young scientist meeting is incredibly well equipped with speakers who are international leaders in their fields, and the three other special meetings – “Actin Dynamics”, “Physics of Cancer” and “Epithelial Differentiation” – provide latest activities in cell biology. These meetings offer, in particular young scientists, the chance to meet top-notch scientists in these various fields; and there are opportunities for them to receive financial support from the DGZ, in case their labs are not able to support their attendance. In any way, members benefit from reduced registration fees.

Referring point five: our society does not publish an official journal. We used to be closely connected to the European Journal of Cell Biology – and the Editors, Stefan Linder, Manfred Schliwa and Sabine Werner, are well connected to the DGZ, and many colleagues on the Editorial Board are members of the DGZ (including myself). Nevertheless, also this publication became an Elsevier journal and does not belong to the society, quite different from the Biochemical Journal and the Journal of Cell Science, which are published by the Biochemical Society and the Company of Biologists, respectively. I have been asked occasionally, if we would not like to make Cell News more professional, having it eventually listed in Pubmed. However, I assume our society is too small to support and run such a project, and secondly the charm and the incredible flexibility is caused by its provisional, spontaneous character, a true “newsletter” aiming to communicate scientific ideas and achievements of our members. And we do not need to care about impact factors but want to spread true interest.

Publish or perish, they used to say

How to publish and even more importantly, how to evaluate the importance of a scientific publication has become a kind of science itself, impactomics, if you want so. At least, this is what “decision makers” want to have: objective criteria, which paper is important and which one is not. And therefore we have experts on IMPACTOLOGY: “Pinpoint your Impact Factor™ to unleash your genius and change the world (at least, your corner of it)” This statement you will find on www.impactology.org. I would not have guessed this item existed. In any way, the word impactology sounds nearly as stupid as “scientology”.

If one dares to criticize the nowadays practice of the high impact part of scientific communication, the sublimation of scientific experimental work in the high impact journals (do they have a “Deep Impact”?), one will be immediately accused of being jealous or mediocre, or that we do not have an alternative to the present system, or that some people do not want us to have alternatives any more. However, what about the recent study in Nature – and their comment on – the “Many landmark findings in preclinical oncology research” that “are not reproducible, in part because of inadequate cell lines and animal models” by C. Glenn Begley and Lee M. Ellis (Nature 483 (2012) 531-533: “Raise standards for preclinical cancer research”, and see also the comment in BMJ 2012;344:e2555 by Nigel Hawkes: “Most laboratory cancer studies cannot be replicated, study shows”). Therefore, what is the “landmarkness” of these articles? Is the standard of these high impact journals not high enough to prevent such duck races and inflation of superiority and novelty? Think for a while: Why not have a super-journal like we had supergroups in the past (“Blind Faith”), and here we go: CNS, actually standing for Cell-Nature-Science, the central nervous system for scientific thought. The editor is “not enthusiastic enough” about this idea? He/she should have a cup of coffee or a dose of Ritalin. But seriously, we will have such a true super-journal soon, made by scientists for scientists: eLife. Fiona Watt, one of the editors of this journal that is supported by the Howard Hughes Medical Institute, the Welcome Trust and the Max-Planck-Society, introduces the special features of the new journal on page 28. All what we can do is: feed it with excellent science.
Introducing eLife

Fiona M. Watt

eLife (http://www.e lifesciences.org/) is a new, top-tier, open-access journal that will cover the full range of biomedical research, from basic cell and molecular biology through to applied, translational and clinical studies. eLife will be highly selective – targeting the very best in scientific advances – but with no special preference given to ‘fashionable’ topics. Randy Schekman (Berkeley) is the Editor-in-Chief and Detlef Weigel (Tübingen) and I are the Deputy Editors.

eLife is being launched this year as a joint initiative of the Howard Hughes Medical Institute, the Max Planck Society, and the Wellcome Trust. Along with a growing number of public and private research funders worldwide, these organisations recognise that the communication of research results is as integral to the research process as the experiments themselves, yet there are a number of flaws in the present system.

eLife seeks to achieve three things:

• To make publishing more efficient, by providing an outstanding service to authors through a swift, constructive, and fair editorial process.
• To drive open access, by providing an outstanding new publishing option for authors that will, in the first instance, be free of charge.
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• Most revised manuscripts will be assessed by the handling BRE member directly, without further outside review.
• The initial decision letter will be published along with the authors’ responses and the accepted version of the article.

One of the reasons that I am committed to making eLife succeed is that I believe that our postdocs and PhD students are being put off careers in science by the brutal process of getting their work published. To be forced to carry out months of additional experiments that end up in supplemental material or, worse, for a hostile reviewer to recommend rejection even when those experiments have been carried out in good faith, is completely demoralising. With the current focus on measuring scientific productivity in terms of numbers of publications and their impact factors, a lengthy delay in acceptance of a paper costs young researchers jobs and fellowships that they are otherwise well qualified to be awarded.

As scientists we need to stand up for fair reviewing, and open access to our research. We need to think creatively about the best ways to present our data for our peers to analyse and use. I believe that eLife will make a difference, and encourage you to submit your best work.

Fiona M. Watt

Director, Centre for Stem Cells and Regenerative Medicine, King’s College London, and Deputy Editor, eLife.
CellNetworks

Science to the power of 5: CellNetworks, the Heidelberg Life Science Cluster of Excellence for all areas in Cell Biology

May-Britt Becker and Oliver Gruss

Established after the first round of applications of the German excellence initiative in 2006, the life-science Cluster of Excellence at Heidelberg University CellNetworks harbors researchers from over 100 work groups in biology and medicine, physics and chemistry. A key concept of CellNetworks has been to bring interdisciplinary research to a new level through novel forms of cooperation between university and non-university researchers as well as by bringing together different scientific disciplines. Having its home base at the BioQuant building in the centre of Heidelberg’s Neuenheimer Feld campus (Fig. 1), the cluster unifies leading researchers from the university, the European Molecular Biology Laboratory (EMBL), the Max Planck Institute for Medical Research, the Max Planck Institute for Intelligent Systems, the German Cancer Research Center (DKFZ), the Central Institute of Mental Health in Mannheim, the Karlsruhe Institute of Technology and the Heidelberg Institute for Theoretical Studies, and is further supported by business partners and the C.H.S.-Foundation.

Hence, unlike other clusters of excellence, CellNetworks manages a broad spectrum of different disciplines, approaches, and mindsets. Not an easy task, to take care of the needs of researchers coming from different institutions. Moreover, CellNetworks aims to provide a framework for the excellent but widespread biomedical science community in Heidelberg (Fig. 2).

CellNetworks: what the logo tells us
Just like its logo, CellNetworks bundles and organizes research in five independent, and closely linked project areas, all building on the technology and methodology platforms:

- Macromolecular Complexes – Biogenesis, Interactions and Regulations;
- Dynamics of Cell Architecture and Interactions with the Extracellular Environment;
- Information Processing in Complex Multicellular Networks;

Figure 1: CellNetworks has its home base in the BioQuant building, an incubator that brings together theoretical and experimental approaches in quantitative research, which was built in 2007 by the state foundation of Baden-Württemberg on the campus for life sciences of Heidelberg University.

Figure 2: Several renowned institutes in Heidelberg and its vicinity generate an excellent research environment.

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CellNetworks

- Alteration of Networks by Infectious Pathogens;
- Central Technology and Methods Platform.

Knowing the scientific landscape in Heidelberg, this may read like a summary of the research topics that are and certainly had already been well represented in Heidelberg before the start of the excellence initiative. CellNetworks therefore defined its goal in reaching the next level: not only to even better support these research areas, but also to foster networking between the individual research groups and to bring the management of basic research to a new level of professionalism. Thus the goal of the cluster goes beyond the support of basic research. It aims at making Heidelberg an attractive science hub for many years to come.

The Excellence Initiative: a unique opportunity for research at Germany's universities

Let's look back to the start of the excellence initiative in 2005, a program supported by German federal and state ministries of education and research to create new funding options for top research at German universities. Due to the increase in the world-wide mobility of research staff it is a central task in academic policy-making to keep "brain circulation" going. It was one important aim of the initiative to boost the attractiveness of working conditions in academic research.

Well, what makes academic jobs attractive? In the early days of CellNetworks, our members asked themselves exactly this question and set up a number of ambitious goals to increase the attractiveness of the Heidelberg campus.

- CellNetworks’ financing model comprises a central budget – big enough to realize very expensive instrumental needs such as specialized electron microscopes, core facilities in mass spec, live cell imaging, crystallography, deep sequencing, high-throughput screens etc. Highly trained technical staff and administrative support as well as scientific management ensure that scientists can focus on science only. The newly built BioQuant offers space for labs, offices and meeting points called "kitchen".

- The excellent financing conditions made it possible to promote young researchers and to train aspiring scientists in special programs for postdocs and junior group leaders. CellNetworks’ Postdoc Program gives all candidates the option to choose a host lab and a work project. Scientific freedom is supported by a personal bench fee.

In addition, goals formulated by CellNetworks were “cross-linking of different fields of life science research in Heidelberg” and “interconnecting research in biology, medicine, chemistry, physics and mathematics”. Obvious aims that everybody wants to reach, you may think. How can you "offer an ideal research climate for interdisciplinary collaboration and scientific cooperation by bringing together researchers"? This may be a key issue given the diverse situation in Heidelberg with institutes nearby but not closely networked. Taking a loan from a map of protein-protein interaction, we may see what CellNetworks reached in five years: Fig. 3a shows the interactions that researchers indicated when analysing their own situation. Each circle represents a cluster member and the colour code relates the researches to one of the five research areas. Lines indicate cooperation in joint research proposals; the length indicates the number of interactions. You can see a high degree of interactions; some interactions are interdisciplinary and some are rather mono-disciplinary nature (Fig. 3).

Five years later, the situation looked very different: not only has the number of groups with collaboration partners in the cluster increased. Stimulated by the recommendations of the Scientific Advisory Board to intensify joint scientific projects, many new ideas emerged which were approached in teams of several laboratories, both within the same research areas, as well as between different areas as interdisciplinary projects (compare Figs. 3a and b).

Empowered by joyful networking CellNetworks’ members played with the creative options and freedom while discussing, looking for supporters, planning, choosing and finally selecting five new emerging colla-
bhorative topics that were called “EcTops” (Fig. 4 and Box). The EcTops were suggested as a new measure for the next application for funding within the framework of Excellence Initiative II by an open call for ideas of interdisciplinary and team-oriented research in Heidelberg. The aim is to enable even risky, early-stage projects that would otherwise be very difficult or even impossible to fund. Resources for the projects will be provided as “global budgets” without fixed assignments, to allow even higher flexibility and to inspire new ideas and new approaches.

Giving research the highest possible degree of freedom was and still is one of CellNetworks’ most crucial aims. Nevertheless, the high degree of freedom in basic research is opposed by often long-term career uncertainties for young researchers. An international view on the question “what makes a location attractive” has recently been given by a study of the Wissenschaftszentrum für Sozialforschung (WZB), which strongly emphasises this issue. The assessment of career progression from enrolment to PhD to professorship was carried out from the perspective of researchers in six countries: USA, Canada, Japan, Sweden, Switzerland and Germany. Four aspects are of major relevance in all six countries:

**CellNetworks: The New EcTops**
- Thermo: Deciphering functional networks from transcription to translation by exploitation of eukaryotic thermophily.
- Cyto: Spatio-temporal coordination of signalling processes.
- Neuro: From synapse to disease: network dysfunction in disorders of synaptic communication.
- Virus: Quantitative analysis and modelling of pathogen replication and spread.
- ncRNA: Non-coding RNAs as versatile regulators of cellular processes.

- Financing and material conditions;
- Conditions for self-determination and creativity;
- Structuring uncertainty in academic careers;
- Qualification for alternative career options.


**Structuring uncertainty in academic careers**

The financing situation and self-determination were successfully targeted by CellNetworks Phase I measures. However, it appears that Structuring Uncertainty and Alternative Career Options are key issues when it comes to choosing where to go next in an academic career, in particular when you look at the German system. Why is that? Especially the German academic system is characterized by a high level of selectivity and career uncertainty. This characteristic uncertainty can be found in the overall staff structure at universities with 83% on temporary contracts (Kreckel et al. 2008: 66f). To put it simply, the German career model does not offer stable positions for junior researchers.

What did CellNetworks do to support researchers at early career stages, and hence, to make Heidelberg an attractive place for young scientists? One of the most appreciated measures turned out to be an international Post-doc program.

**THE INDEPENDENT POSTDOC**

Right from the start, CellNetworks identified the recruitment of international postdocs as one of the bottlenecks in the staffing
CellNetworks

situation and therefore launched a tailored postdoc program. Twenty-six postdoctoral fellows were recruited to 19 groups in 8 calls for application (Fig. 5). The selection is performed by the CellNetworks Postdoc Selection Committee in a 2-step review process. In step 1 an online application including a project proposal to work on in the CellNetworks host laboratory is evaluated. Candidates need to discuss the proposal with the future host and get the host’s agreement to application. Step 2 consists of a personal interview with the committee and full-day interviews with the prospective host group. The selection of successful candidates is based primarily on scientific excellence. The committee ranks the applicants and decides on the final list. The recruitment of applicants from abroad is strongly favoured and for German candidates mobility is a prerequisite for acceptance. A total of 26 CellNetworks postdocs have been recruited so far, and 16 of the 77 candidates who were interviewed but not selected (from an initial group of about 300 applicants) were still hired by their host laboratory under different funding schemes. Thus, the program serves as efficient recruitment platform. The group of CellNetworks postdocs is highly international with only 27% of all postdocs coming from a lab in Germany (Fig. 5). Our international marketing activities focus on enhanced visibility at recruiting events and scientific meetings.

THE TENURED PI

The biggest hurdle during a career in basic science is certainly to reach the level of a tenured PI. In Germany, the vast majority of positions are embedded into a professorship at a German university that is usually advertised openly. The alternative that the American system offers, to become a permanent PI at the place where you started your career, is rather an exception. Creating the possibility of a tenured position for the best young researchers was a key item on the CellNetworks agenda.

In consultation with CellNetwork members the problem has now even been implemented in the “Zukunftskonzepte” funding application of Heidelberg University. The rectorate decided to implement a “Start-up Professorship” program, where group leaders are initially appointed for 5 years with the option of extending for 6 more years after a competitive international review. For the extension period, the group leader will be promoted to a temporary professorship with all academic rights. This appointment does not require a free professorship as the position can be funded by tapping-up the group leader position during the period of the professorship.

Reasons to choose CellNetworks in Heidelberg for the next step in your career:

Heidelberg JUNIOR GROUP LEADERS are sought after as TENURED PIs

What does the situation look like in the cluster? During the first funding phase, 12 CellNetworks junior group leaders were recruited as professors or received equivalent positions outside Heidelberg, and 5 junior group leaders were appointed to a professorship at Heidelberg University. Among today’s members of CellNetworks there are a total of 22 independent junior group leaders representing almost all institutions of the cluster. This highlights their essential contribution to the research program. Their career uncertainty remains one of the biggest challenges for the cluster. However, it seems that choosing Heidelberg for an early career stage is not a bad option at all. Even though not all juniors will have the opportunity to stay, they will have very good chances to be appointed to a permanent professorship at other excellent places in Germany or abroad (Fig. 6).

Summary

After six years of funding the CellNetworks researchers look back to re-estimate the success and the development of the self-built

structure of their cluster of excellence. The main goal was to make Heidelberg an even more attractive place to do research. And we do not keep this information secret. Instead, we actively promote the international visibility of Heidelberg’s life sciences. There is no doubt that the cluster succeeded to provide an excellent surplus of infrastructure. Contacts between groups in Heidelberg were fostered and new collaborations initiated not only pro forma but yielding true collaborative projects. The newly funded Collaborative Research Center (SFB 1036) on “Cellular Surveillance and Damage Response” is the youngest result of a group of people who have teamed up in CellNetworks.

The Cluster CellNetworks will face future challenges but it appears that it is on a good track and well prepared to profit from the scientific prospect offered by the excellence initiative.

May-Britt Becker and Oliver Gruss

Figure 6: Success in reaching the next career step: 17 junior group leaders (JGL) from the starting group of 20 received a tenured position. Out of the junior group leaders with a membership start between 2007 and 2011 already 9 hold a tenured position.
Forming sarcomeres – with a little help from the cytoskeleton

Elisabeth Ehler

The contractile proteins actin and myosin are arranged in striated muscle cells in a paracrystalline fashion to sarcomeres, yielding regular stripes that can even be distinguished by phase contrast microscopy. Yet, the static nature of this regular arrangement, as suggested by light and even more so by electron microscopy is hugely misleading, because not only can the protein composition of the sarcomeres be adapted, depending on the work load (e.g. different myosin isoforms), but there is also continuous turnover and maintenance. With isotopes from the early 80ies has suggested an average half-life for actin and its associated proteins that is in the range of half a week (Martin, 1981). My lab is interested how these extremely regular structures are assembled during embryonic development and how the maintenance is affected in heart disease.

The sarcomere is the basic unit of a myofibril and is defined as the region in-between two Z-discs. The Z-discs anchor the actin filaments, which together with their associated proteins tropomyosin and the troponin complex, make up the so-called “thin” filaments. The thick filaments are composed of myosin and its associated proteins (e.g. MyBP-C) and are arranged in a bipolar fashion, with the myosin tails making up the core of the filament and the heads being located at both ends, where they interact with the actin. Elastic filaments, composed of the protein titin (also called connectin), run from the Z-disc to the middle of the sarcomere, where a structure called the M-band integrates them with the thick filaments (for schematic drawing see Figure 1A).

The “sarcomeroskeleton”

Titin’s N-terminus is anchored at the Z-disc and crucial for Z-disc assembly and width (Young et al., 1998). Since from there this 1 micrometre long molecule stretches through half a sarcomere to the M-band (Fürst et al., 1988), it has been suggested for a long time to serve as a ruler for the sarcomere (Trinick, 1994) and as a blueprint for the integration of a multitude of other proteins of the sarcomere (Lange et al., 2002; Linke, 2008). Genetic evidence for titin’s crucial role in sarcomere assembly has come from a multitude of model systems (van der Ven et al., 2000; Xu et al., 2002) and also been probed further by genetically altered mouse models and cardiomyocytes (Musa et al., 2006; Weinert et al., 2006). Due to its sheer size titin has been an absolutely daunting task to sequence (Labeit and Kolmerer, 1995) and it is probably due to this that it has taken until very recently to establish mutations in the titin gene (TTN) as a prevalent cause for dilated cardiomyopathy (Herman et al., 2012). While titin explains how the longitudinal axis of the sarcomere might be organised and assembled during myofibrillogenesis, there is currently no direct proof, which molecules might be crucial for the assembly of the two major transverse structures in the sarcomere, the Z-disc and the M-band. These structures can be clearly distinguished in every mature sarcomere in cross-striated muscle in situ and in cultured myocytes (Figure 1B) and are characterised by the “marker” proteins sarcomeric alpha-actinin and myomesin, respectively, which have until now been identified in every vertebrate sarcomere as soon as they have been formed. However, genetic evidence for their essential role is still lacking, apart from observations that point mutations may be causative for HCM (Chiu et al., 2010; Siegert et al., 2011). We wanted to test the hypothesis that titin, sarcomeric alpha-actinin and myomesin may together constitute a kind

Figure 1: A: Schematic drawing of a set of sarcomeres above an isolated adult cardiomyocyte (nuclei shown in black). Thick (myosin) filaments, thin (actin) filaments and elastic filaments (titin) are represented in different shades of grey; the polarity of the actin filaments is indicated by chevrons showing the barbed respectively the pointed end in the top left hand side. ID intercalated disc; T junctional (Bennett et al., 2006).

The two major transverse structures, the Z-discs (in red) and the M-band (in green) are indicated. B: Neonatal rat cardiomyocyte in culture, showing the two major transversal structures that organise the sarcomere, the Z-disc (stained for sarcomeric alpha-actinin, in red) and the M-band (stained for myomesin, in green). Nucleus stained with DAPI in blue. Bar represents 10 micrometres.
of sarcomeroskeleton that is necessary and sufficient to integrate and maintain the other components and started our experiments in a cell culture model system. Cardiomyocytes are hugely specialised cells and unfortunately no cell line exists that would fully represent them in all their facets. HL-1 cells, which were established from atrial tumours, are extremely useful, because they have all characteristics of a cell line and express the set of transcription factors that give it cardiomyocyte identity (Claycomb et al., 1998). However, from a morphological point of view they represent an early developmental stage of cardiomyocytes, since they share their epithelioid shape with cardiomyocytes in the early embryonic heart (Hirsch et al., 2006; Manasek, 1968) with cell-cell contacts running all around the plasma membrane and myofibrils, which are usually very thin and immature-looking and located preferentially at the periphery of the cells (Figure 2, top row). Primary cultures of neonatal rat cardiomyocytes (NRC) have a more mature morphology, with intercalated disc-like structures restricted to the sites of cell-cell contact and a dense network of well-differentiated contractile myofibrils throughout their cytoplasm (Figure 2, bottom row), but obviously have all the drawbacks of primary culture, such as limited life-span and inefficient yields during transient transfection. We devised an experimental technique that allowed us to knockdown the expression of our protein of interest and at the same time to visualise the targeted cells by combining a Hs cassette driving the shRNA (Brummelkamp et al., 2002) with a CMV promoter driving GFP-expression on the same plasmid (Iskratsch et al., 2010). Confocal microscopy analysis of NRC eight days after transfection with these plasmids, shows the naughty habit of GFP to accumulate in the nucleus and also to associate with the actin cytoskeleton of PFA-fixed cardiomyocytes, but the GFP overexpression appears to have no deleterious effect on the myofibrils (Z-discs shown in blue, stained with antibodies against sarcomeric alpha-actinin; M-bands shown in red, stained with antibodies against myomesin; Figure 3 top row). Expression of the shRNA that is supposed to specifically target sarcomeric alpha-actinin expression, at the first glance also has no discernible effect, until one notices the lack of alpha-actinin signal in the periphery of the cells, where the regions of novel myofibrillogenesis are situated (Figure 3 middle row, arrows). Knockdown of myomesin expression for 8 days has a dramatic effect on myofibril integrity and results in residual sarcomeres with large gaps in-between that are connected by (GFP-decorated) actin cables (Figure 3 bottom row). Since NRC already have myofibrils when they are isolated and plated into the culture dish, these experiments obviously do not tell us anything about the role of alpha-actinin and myomesin in de novo myofibrillogenesis, but they highlight extremely interesting differences between those two transverse structures of the sarcomere in myofibril maintenance. Once the Z-discs are formed they seem to be very stable structures that are resistant to several types of interference, such as e.g. the downregulation of the expression levels of a potentially crucial structural strut such as alpha-actinin. Similar observations were made, when cardiomyocytes in culture were incubated with actin depolymerising agents such as e.g. cytochalasin D. While an intact actin cytoskeleton is absolutely crucial for the isolated cardiomyocytes to attach to the culture substrate, once they have attached their mature myofibrils seem to be totally resistant to cytochalasin action and it is only the peripheral actin cytoskeleton than gets depolymerised (Rothen-Rutishauser et al., 1998). This is the same actin cytoskeleton that together with the N-terminus of titin and with alpha-actinin seems to form the nuclei for myofibrillogenesis (Ehler and Gautel, 2008). This suggests that these structures are more transient and hence more prone to suffer from interference either with the integrity of the actin cytoskeleton or with sarcomeric alpha-actinin expression levels. The M-bands on the other hand seem to be the place in the sarcomere that becomes vulnerable to contractile activity, once there is interference with the expression levels of one of its potentially essential constituents. The force-managing role of the M-band has been proposed a while ago (Agarkova and Periáñ, 2003) and also the observations that there is a signalling cascade emanating from the M-band to the nucleus,
which is activated mechanically (Lange et al., 2005), provides further evidence for a more active role of the M-band in dealing with contraction management compared to the more passive role of the Z-disc in comparison. These conclusions are supported by electron microscopy data that show almost unchanged arrangements of the Z-discs and dramatically affected M-bands in activated muscle (Horowits and Podolsky, 1987). However, the genetical experiments that would provide unequivocal proof for the crucial role of a sarcomeroskeleton are so far still outstanding and not exactly facilitated by the fact that both alpha-actinin and myomesin appear to be among the parts of the zebrafish genome that has been duplicated during evolution (Bakkers, 2011).

**How to maintain actin filament length in the contracting sarcomere?**

Traditionally, actin (thin) filaments in striated muscle are seen as inert strictly parallel filaments of a clearly defined length that are arranged in this way to maximise contractile force output when interacting with the heads from the biopolar myosin (thick) filaments. They are capped by CapZ at their barbed end (located in the Z-disc, see Figure 1A) (Schafer et al., 1995) and by tropomodulin at the pointed end, located towards the M-band (Gregorio et al., 1995). However, already the early protein turnover experiments suggested that there might be more to an actin filament than just being a tropinin/tropomyosin decorated piece of string (Martin, 1981) and also ultrastructural studies around the same time indicated that actin filament length may actually depend on the exact muscle type that is studied (Robinson and Winegrad, 1979). These differences were then attributed to different splice variants of nebulin, a giant protein that runs along the actin filament in striated muscle (Trinick, 1994), however, since only minor amounts of nebulin are expressed in the vertebrate heart (Bang et al., 2006; Kazmerski et al., 2003; Witt et al., 2006) and high resolution experiments have shown that nebulin actually does not extend to the very end of the actin filament (Littlefield and Fowler, 2008) there must be a different mechanism, how the length of a thin filament is maintained in muscle. Recently it has also been shown that the capping proteins CapZ and tropomodulin are not as tightly bound as initially assumed and that there are also other players around at the pointed end, such as e.g. leiomodin (Chereau et al., 2008). In the sarcomere during early embryonic development tropomodulin seems to be a leaky capper, allowing for actin filament extension and no leiomodin is expressed. In mature sarcomeres tropomodulin caps the pointed ends more tightly and leiomodin may associate with a distinct population of actin filaments and allow for addition of more actin monomers to the pointed end (Skwarek-Maruszewska et al., 2010; Tsukada et al., 2010; reviewed in Dwyer et al., 2012).

The most attractive hypothesis for thin filament length regulation has been proposed by Littlefield and Fowler (Littlefield and Fowler, 1998; Littlefield and Fowler, 2008) and postulated an activity driven mechanism, where actin filaments that interact productively with myosin heads are maintained at a stable length, while actin filaments that do not can either shrink or even extend. Whether these two actin filament populations can be directly correlated to the contractility dependent actin dynamics observed by the Lappalainen group in cultured cardiomyocytes, remains to be determined (Skwarek-Maruszewska et al., 2009). Embryonic cardiomyocytes in situ at early developmental stages do display actin filaments of different lengths in their sarcomeres, which are also distinct in their actin isoform composition. Smooth muscle alpha-actin, which is still expressed at early stages of myofibrillogenesis in cardiomyocytes, runs in an apparently continuous fashion, probably representing overlapping actin filament populations from adjacent Z-discs, while cardiac alpha-actinin shows clearly distinct I-bands (Ehler

**Figure 3:** Neonatal rat cardiomyocytes after 8 days of shRNA mediated knockdown of the expression of sarcomeric alpha-actinin (middle row) or myomesin (bottom row), the control shRNA is shown in the top row. Targeted cells were identified by their expression of GFP, which is encoded on the same plasmid as the shRNA. Knockdown of sarcomeric alpha-actinin has little effect on the cellular morphology, apart from reduced presence of alpha-actinin in the peripheral regions of myofibrillogenesis (arrows). Knockdown of myomesin expression leads to a fragmentation of the myofibrils (arrowheads) compared to neighbouring control cells. Bar represents 10 micrometres.
et al., 2004). In addition to the muscle actins there is also a certain amount of expression of cytoskeletal actins in myocytes, whose role only begins to be appreciated (Belyantseva et al., 2009; Prins et al., 2011; reviewed in Dwyer et al., 2012).

Old hands in the actin game at hand to aid thin filament formation

The most exciting recent developments in the field of the muscle actin cytoskeleton have taken their inspirations from decades of study on the regulation of actin in cell migration. Well known players such as N-WASP or a member of the formin family, FHOD3, have now turned out to be important for thin filament synthesis and maintenance in myocytes (Iskrets et al., 2010; Takano et al., 2010). N-WASP appears to be crucial for addi-
tional myofibril synthesis during IGFs-induced skeletal muscle hypertrophy (Takano et al., 2010). The knockdown of FHOD3 expression in cultured cardiomyocytes leads to myofibril breakdown and FHOD3 also greatly facilitates actin filament synthesis following latrunculin-induced depolymerisation in NRC (Iskrets et al., 2010). In both cases, an actual role in the process of de novo myofibrillogenesis remains to be proven and there are also gaps in our knowledge, for example the identity of the downstream actin elongation factor from N-WASP and the exact role of FHOD3 in the actin filament formation due to the lack of proper actin biochemistry experiments. However, these observations highlight the importance of a shift from a static view of the sarcomere and the thin filaments to a more dynamic view and it is to be expected that several other proteins that are important in the regulation of actin filaments during cell migration, will move also into the limelight of the muscle cytoskeleton field. For example, ROCK is expressed at high levels in the embryonic heart and crucial for embryonic heart function (Wei et al., 2001; Zhao and Rivkees, 2003) and its expression levels get downregulated around birth. However, ROCK expression is upregulated in hypertrophic growth in the heart, i.e. when there is a need for additional actin filament synthesis (Ahuja et al., 2007). A combination of the cutting edge tools, such as e.g. Lifeact (Riedl et al., 2008), which the cell migration field has at its disposal with life imaging in cardiomyocytes should yield exciting results that will help to advance the field. Initial localisation studies of Lifeact in fixed cardiomyocytes have revealed incorporation predominantly into actin fibres at the periphery of the cells, again indicating a higher actin turnover rate that in the mature sarcomeres in the middle (Figure 4). However, there is also incorporation of Lifeact into the myofibrils and it will be exciting to investigate it more closely in living cardiomyocytes in the future. In conclusion, there is more to a sarcomere than the contractile proteins actin and myosin and more dynamic cytoskeletal events take place in a muscle cell than just contraction.

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Tribes, Territories, Migration, Displacement. The use of cell labelling to study tissue dynamics in muscle and pancreas

Harald Jockusch, Volker Kroehne and Daniel Eberhard

Introduction
As exemplified by neural crest, skeletal muscle and germ cells, the development of the vertebrate body involves migration of precursor cells over considerable distances. This was clearly demonstrated in quail-chick transplantation chimeras in which a species difference in the morphology of the nuclei served as a marker to distinguish donor from recipient cells. The underlying assumption was that the mixing of different species would not interfere with the basic processes of embryonic development (Le Douarin and Teillet, 1974).

Here we give a review on the application of cell labelling to the analysis of muscle development and pathology, as well as the development of the pancreas. Skeletal and cardiac as well as smooth muscle have been investigated. All experiments were performed in mice but in some cases rat tissue and a permanent myogenic mouse cell line were used.

Where do you come from?
Cellular labels of origin
Clonal expansion and intermingling, migration and settling of cells, as well as acquisition and defence of tissue territories are important processes in the developing metazoan body. In order to follow these during normal development and in pathological situations, to track the fate of clones and polyclones or the dispersion of single cells, a label is required. In some cases, naturally migrating or transplanted cells carry their own label due to a stable differentiation marker absent from, and not influenced by, their environment, as alkaline phosphatase in the case migrating germ cells or α-myosin heavy chain as in case of ectopically transplanted atrial tissue (Jockusch et al., 1986; Jockusch and Heimann, 1991). The underlying assumption of such experiments is that, with respect to the label, there is no mutual influence between the traced cells and the cells by which they are surrounded.

For the study of clonal expansion, cell mixing and migration within a given organ or tissue, a “label of origin” rather than of differentiation is required. Fluorescent dyes have been used to artificially label cells, but these would be diluted in proliferating cell populations.

Ideally, a label of origin should be
– cell-autonomous and at least somatically heritable
– able to distinguish cells of the same differentiation state within an organism
– developmentally and physiologically neutral, i.e. should not result in a selective disadvantage (or advantage, cf. Augustin et al., 1998) over unlabelled cells.

Already in the early days of experimental embryology the importance of distinguishing donor from host in transplantation experiments was recognized. Pigmentation differences between newt species were used by Hans Spemann and Hilde Mangold to decide between contribution to, and induction of, axial organs in amphibian development (Spemann and Mangold, 1924).

From the dark ages to the age of green fluorescent enlightenment
A cellular label of origin might be based on a difference in DNA, protein or oligosaccharide.

A genetic variation of an oligosaccharide side chain, as monitored by lectin binding, has been used to analyse the clonal origin of intestinal crypts in mouse chimeras (Schmidt et al., 1988). However, the applicability of a surface oligosaccharide is limited to a few tissues.

A DNA label like the Y chromosome in sex chimera (e.g. Grounds et al., 1991) or high copy number transgenes (Augustin et al., 1998) have the disadvantage that they require in situ hybridization to be monitored; this method is extremely rough on the intactness of histological sections and requires numerical corrections for partially lost nuclei. Only half of randomly aggregated embryos will yield XX ↔ XY sex chimeras. Unless one wants to study gonadal development, sexual chimerism is usually neglected in the study of chimeras. With protein labels, the simplest cases are genetic variants of a naturally occurring, ubiquitously “expressed” protein, e.g. an enzyme. The best known example is the cytoplasmic soluble enzyme, glucose phosphate isomerase (GPI), which comes in electrophoretically distinguishable alloforms. Alternatively, a protein label might be introduced via a stably integrated gene from another organism, like β-galactosidase (coded for by the E. coli gene LacZ) or jellyfish green fluorescent protein (GFP) and its artificial
variants). Highly soluble and diffusible proteins like GFP distribute over a considerable distance within the labelled cell, including cytoplasmic processes. For certain purposes a protein with a nuclear localization signal attached to its sequence (like nLacZ) might be a useful label as it would be localized in nuclei in the neighbourhood of the nucleus that carries the nLacZ transgene. Depending on the question to be answered, a ubiquitous or cell-type specific expression of the transgene might be of advantage.

In the seventies, the allvariants of the di- meric cytoplasmic enzyme glucose phosphate isomerase (GPI AA and GPI BB) became popular in mouse embryology and transplantation analysis. The enzyme test on electro- pherograms is highly sensitive but requires extraction of the tissue. To our knowledge, attempts to produce GPI alloform specific antibodies have not been successful. Yet, frozen sections of skeletal muscle could be divided and analysed so that a limited morphological analysis of parental contributions in embryo chimeras and in transplantation experiments were feasible. For the analysis of skeletal muscle a useful feature is the fact that the GPI AB heterodimer indicates that nuclei of different origin must have resided in the same, heterokaryotic cytoplasm. This is the case in skeletal muscle after co-fusion of myoblasts of different parental origin (Fuchtbauer et al., 1988; Partridge et al., 1989).

Age of enlightenment: enter GFP and its variants, and an anecdote

The introduction of the jellyfish green fluorescent protein (GFP, 238 amino acids, Mr = 26.9 KDa) as a cellular and molecular (as in fusion proteins) marker dramatically simplified matters, caused an explosion of publica- tions based on that label and made the dis- co verers Nobel laureates in chemistry (2008). Both, in whole mounts and in sections, la- belled cells can be scored by UV fluorescence microscopy; in contrast to LacZ (βgal), no histochemical staining reaction is required.

Originally, we wanted to use Okabe’s “green mice” (Okabe et al., 1997) that had been described as “ubiquitously expressing” enhanced GFP (eGFP) controlled by the chicken β-actin promoter, to analyse chimeric CNS and testis of the mouse mutant wobbler (Augustin et al., 1998; Schmitt-John et al., 2005). Disappointingly, the particular strain of green mice that we had ob- tained via Professor Melitta Schachner in Hamburg expressed eGFP extremely poorly in CNS and testis. On the other hand, expression in all three muscle types, skeletal, cardiac and smooth, as well as in pancreas and kidney was very high. Upon deflecting the skin from a “green mouse”, muscles appeared greenish in daylight even without UV illumination. In cross-striated muscles, only muscle cells were eGFP positive whereas intervening connective tissue was negative. Thus the “Hamburg strain” of Okabe’s green mice seemed to provide an ideal mate- rial for experimental studies of cross-striated muscle, results of which will be reviewed in this article.

Evasive Green

The father of green mice, Okabe, suggested for their study to fix whole organs in formal- dehyde (“bath fixation”) prior to sectioning. Yet, a detailed study of skeletal muscle development or regeneration usually involves
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<td>T</td>
<td>GPI alloforms, microanalysis of sections</td>
<td>Hereditary myotonia is muscle intrinsic; secondary effects of myotonia (e.g., parvalbumin concentration) are dependent on muscular environment.</td>
<td>Füchtbauer et al., 1988</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>T</td>
<td>eGFP, nLacZ double and complementary</td>
<td>Migration of muscle precursor cells across fascial and muscle borders.</td>
<td>Fig. 1 Jockusch and Voigt, 2003</td>
</tr>
<tr>
<td>Myogenic cells (permanent cell line)</td>
<td>T, using artificial matrix</td>
<td>eGFP (host)</td>
<td>C2C12 myogenic cells fuse to form parallel myotubes in ordered collagen matrix. Regenerated graft contracts upon direct stimulation.</td>
<td>Fig. 2 Kroehne et al., 2008</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>X</td>
<td>eGFP, nLacZ</td>
<td>Large scale left-right differences in parental contributions, indicating largely coherent growth of (poly-)clones; mixing at borders resulting in co-fusion. In very small muscles (toe muscles) segregation into monophenotypic muscles due to bottleneck effects.</td>
<td>Eberhard and Jockusch, 2004</td>
</tr>
<tr>
<td>Myocard</td>
<td>X</td>
<td>eGFP</td>
<td>Moderate number of progenitor clones; patches with irregular shapes of highly variable sizes; coherent expansion with limited mixing at the borders.</td>
<td>Fig. 3 Eberhard and Jockusch, 2005</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>X</td>
<td>eGFP</td>
<td>Small clones in gastric and intestinal walls; correlated location between two muscle layers.</td>
<td>Fig. 4 Eberhard unpubl.</td>
</tr>
<tr>
<td>Pancreas</td>
<td>X</td>
<td>eGFP</td>
<td>Coherent expansion of acinar (poly-) clones leads to large patches. Acini are polyclonal in neonates, but monophenotypic in adult mice. Islets are oligoclonal at both stages with developmental independence from surrounding acinar tissue.</td>
<td>Fig. 5 Eberhard and Jockusch, 2010</td>
</tr>
</tbody>
</table>

a distinction between, and analysis of, fibre types: fast vs. slow, glycolytic vs. oxidative etc. Fibre types are defined by myosin heavy chain expression and metabolic enzyme patterns. These properties are monitored by enzyme and immuno-histochemistry on adjacent unfixed frozen sections. An unfixed muscle section, however, would lose GFP immediately, due to GFP’s extreme solubility. Even fixation of a section with a drop of formaldehyde (“drop fixation”) will fail, due to nearly instantaneous loss of GFP by diffusion, as predicted by Okabe (“don’t even think about this”). To solve this problem, we chose a method that had been successful with another small, extremely soluble and stable protein, parvalbumin (Füchtbauer et al., 1991), namely fixation of dry frozen sections in the gas phase at -20°C, with formaldehyde vapour (See Figs. 1, 2 and Jockusch et al., 2003; Jockusch and Eberhard, 2007).

Transplantation and embryo chimeras: invisible borders made visible
The experiments on mice performed over the years involved myocardial tissue, skeletal muscle, smooth muscle, and the pancreas. Basically two types of experiments were performed to create chimeras, i.e. individuals composed of cell populations of different parental origin: Transplantation (T) of donor or graft (g) skeletal muscle or myogenic cells and embryo chimeras (X) produced by the aggregation of 8-cell embryos (McLaren, 1976), in which all tissues could be analysed which express the label (Table).

You can’t blame the environment for myotonia
Myotonia is a rare human hereditary disease which is characterised by hyperexcitability of mature skeletal muscle. This occurs in dominant Thomsen and a recessive Becker form. A goat model and, later on, a mouse model became available to experimentally study this disease, and the myotonic ADR mouse was finally instrumental in identifying the molecular basis of these myotonia: loss-of-function mutations in the gene for the muscular chloride channel (Steinmeier et al., 1991). However, before that was
known, the muscle intrinsic origin of the disturbed excitability (as opposed to a neurogenic or humoral cause) was demonstrated by muscle transplantation in conjunction with a quantitative evaluation of the GPI alloforms that distinguished host from donor (Füchtbauer et al., 1988).

**Skeletal muscle: immigration and emigration**

In the discussion on a possible cure of the fatal hereditary Duchenne muscular dystrophy (DMD) the issue of survival and migration of muscle precursor cells plays an important role. We used whole muscle grafting with either double or complementary labelling by eGFP and nLacZ to study immigration from graft into host and, conversely, emigration from host into graft. In this situation fascial borders and borders between adjacent muscles are crossed, with co-fusion occurring between donor and host muscle precursor cells. Migration, however, is slow, amounting to only one to two millimetres in 10 to 11 weeks. The migration activity and crossing of borders are probably stimulated by the traumatic situation shortly after transplantation (Fig. 1).

**Settlers from another world: are they helpful?**

Using an eGFP host label we have analysed the regenerative potential of tissue engineered myoblast/matrix constructs upon grafting into empty beds of anterior tibial muscle (Kroehne et al., 2008). Permanent myogenic cells (C2C12) were cultured using artificial scaffolds (collagen sponges, CS) consisting of collagen-I with a parallel pore geometry and subsequently grafted into the muscle bed of eGFP-transgenic nude mice (Fig. 2). Here we used a 'negative labelling' of the donor cells i.e. absence of the eGFP label (cf. Jockusch and Voigt, 2003). In conjunction with cell fusion during myogenesis and the high diffusibility of eGFP within a given muscle fibre (Jockusch and Voigt, 2003) the transgenic eGFP labelling of host muscle provides an extremely sensitive measure for host contributions to the regenerating muscle fibres at the graft site (Fig. 2B). Fibres of mixed donor-host origin can be recognised by their intermediate levels of eGFP (Fig. 2C).

**Mice with four parents**

The subsequent findings are based on embryo chimeras, i.e. mice that were derived from the aggregation of two embryos ("tetraparental mice"), in our case distinguished by one or two labels, eGFP and nLacZ. As in transplantation chimeras, eGFP surface fluorescence allows for a preliminary macroscopic evaluation. Initially, a standard aggregation embryo is composed of equal numbers of cells derived from the two pairs of parents. But upon the specialisation into trophoblast and inner cell mass, with the embryo proper originating from the latter, a random sampling leads to different proportions of the two parental contributions. Although a chimeric embryo starts out with a doubled cell number, sizes are down-regulated to normal and, except in the gonads of sex chimeras, no abnormalities are observed as a result of chimerism. A chimera may be "balanced" with contributions from 0.4 to 0.6, or "unbalanced" with one partner represented by only a fraction of 0.1 to 0.2, or intermediate. Resulting areas of uniform phenotype will represent clones, or, more commonly, oligo- and polyclones, termed "patches" (West, 1999). Mono- and oligoclonal are more likely to be Figure 2: Use of an eGFP host label to analyse the contribution of in vitro engineered myoblast-matrix grafts to muscle regeneration.

A: Scanning electron micrograph showing the structure of the collagen sponge (CS) used as matrix for the engineering of skeletal muscle. The orientation of the extended pores (vertical in the micrograph) defined the longitudinal axis of the internal structure of the sponge scaffold. In the transverse direction (horizontal in the micrograph) there are interconnecting bridges and holes. B, B', C, C', C'': Donor and host contributions to regenerated muscle derived from a graft of C.C..CS (8 mm x 3 mm x 2.5 mm) seeded with $1.5 \times 10^5$ cells that has been cultured for 7 days under proliferation-inducing and for 5 days under differentiation-inducing conditions. Analysis 14 days after surgery. B and B', adjacent cross-sections of the regenerated graft; B, formaldehyde vapour fixed to show eGFP fluorescence (green); B', unfixed for immunostaining of myofibrillar myosin (red). Original host muscle (h) is situated to the left, the regenerated graft (g) to the right. Regenerating host muscle fibres have invaded the middle and right outer portion of the graft, but myotubes and myofibres in large areas are eGFP negative and hence derived from donor-C.C. cells only. C, C': Enlargements of areas indicated by dotted outline in B and B', respectively. C'': overlay of C and C'. In C, regenerated myofibres of host origin are identified by high eGFP fluorescence and centrally located nuclei. They are surrounded by high numbers of eGFP-negative and hence donor-derived immature muscle fibres and myotubes (C') and (C''). Scale bar, 100 μm in A; 200 μm in B and B'; 50 μm in C, C' and C'', respectively. (Kroehne et al., 2008)
recognised in unbalanced chimeras. In order to get important global information on the issues of clonal expansion vs. intermingling, it is not necessary to deal with single clones.

Conquering a large territory: competition among tribes or intermingling

The formation of skeletal muscle involves migration of muscle precursor cells from the somites near the dorsal midline down to the sides and into the limbs. During differentiation they fuse to form myotubes and, finally, mature muscle fibres. Macroscopic inspection of the back of unbalanced chimeras showed asymmetric and unevenly distributed eGFP positive muscle portions; this resulted from the efficient separation between left and right muscle masses along the midline. Even within muscles on one side and within limb muscles dramatic differences in the eGFP content were observed. This shows that there is no fine-grained intermingling of muscle precursor cells during myogenesis. With the exception of the small interosseus toe muscles total absence of eGFP from muscles was not observed; even quasi-monophenotypic eGFP negative extra-ocular muscles showed a very low level of eGFP, indicating co-fusion of a few eGFP positive myoblasts. The eGFP negative toe muscles are probably explained by a founder effect, sampling of a small number of settlers from a large mixed population on the move (Eberhard and Jockusch, 2004).

Myocardial histogenesis – how many ancestors?
The complications of extensive migration and cell fusion do not occur in myocardial histogenesis. Heart development might lead to a disperse distribution of many small patches (a pepper-and-salt pattern) either due to a large number of progenitor cells and/or to extensive local cell mixing. Or, alternatively, with a limited number of progenitor cells and coherent expansion of (oligo-clone) patches, macroscopic patches might be recognised. Chimeras were either of the eGFP ↔ 0 type, i.e. one aggregation partner was labelled with eGFP, the other unlabelled; or of the GFP ↔ 0 nlacZ type, i.e. with two complementary labels. It became clear that cardiac morphogenesis does not result in a fine grained “salt-and-pepper” distribution of the descendants of primordial heart cells; rather, relatively large patches are observed, especially in the ventricle, indicating coherent clonal expansion, whereas the patch patterns are more complicated in atrium and septum. By 3-dimensional reconstruction it
was shown that “very small patches” were usually protrusions of large patches, disconnected by sectioning. Patches have irregular shapes that seem to follow tension vectors, especially in the apex of the ventricle. Their borders are frayed indicating local mixing of myocytes at territorial borders between oligoclines (Fig. 3 and Eberhard and Jockusch, 2005).

Social stratification in intestinal smooth muscle

Smooth muscle occurs in layers and cords of varying thickness and orientation forming the walls of hollow organs including the gastrointestinal tract where contractility and stretch resistance are required for function. In the intestinal walls, the external muscular layer (muscularis externa) consists of inner circumferentially (stratum circulare) and outer longitudinally oriented smooth muscle layers (stratum longitudinale, Gabella, 1950). Studies in mouse, rat and chick have shown that the outer longitudinally oriented muscle develops after the formation of the circular layer (Masumoto et al., 2000; Gabella, 2002) and histological studies in the chick have suggested that the longitudinal layer is established by radially oriented muscle fibres protruding from the inner circular muscle anlage (Masumoto et al., 2000). We used GFP ++ 0 mouse aggregation chimeras to analyse the clonal relationship between these two muscle layers of the muscularis externa (Fig. 4, Eberhard unpublished). In chimeric gastrointestinal walls, the eGFP label revealed spindle-shaped bundles of 50-100 eGFP-positive smooth muscle cells in the circular layer and, spatially related to these, thin rows of eGFP-positive myocytes in the longitudinal layer (Fig. 4A). The overlap of patches in the circularly oriented and longitudinal layer observed here suggests a direct clonal relationship between the two adjacent smooth muscle layers as opposed to an independent de novo formation of the longitudinally oriented muscle (Fig. 4D).

Territories and migration in the pancreas

The pancreas has a lobular architecture and consists of mainly epithelial tissues. It comprises exocrine (multicellular acini and ducts secreting and transporting enzymes to the gut) and endocrine components (multicellular islets of Langerhans releasing hormones like insulin into the blood stream). The lobular pancreas emerges from small endodermal buds that subsequently branch, leading to the development of a network of acinar structures connected by ducts (Slack, 1995; Eberhard and Jockusch, 2010).

We studied mouse chimeras to analyse the nature of clonal growth during pancreas development and to find out whether acini and islets were mono- or polyclonal. Fortunately

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**Figure 4: Chimerism in the smooth muscle layers of the small intestine in eGFP ++ 0 chimeras.**

A, B: Analysis of a three week old chimeric intestine by confocal laser microscopy in an unbalanced chimera. A: Distribution of eGFP-positive patches in circular and longitudinal sheets (arrows); projection through an image stack of 42 optical sections comprising 50 µm depth. B: Reconstruction of overlaps framed in A with eGFP-positive areas color coded for relative depth (rotation 0° and 40°) showing patches in the outer longitudinal and inner circular smooth muscle layer, which “touch” in the overlapping region. Note the smooth muscle cells that are interwoven with in the longitudinally oriented myocytes (arrow). L, longitudinally and C, circularly oriented smooth muscle layer. C, C: Smooth muscle cell patches in transverse section of the muscularis externa in a three week old low eGFP chimera. eGFP fluorescence (green) combined with nuclear stain (Hoechst, blue) in C, and smooth muscle α-actin stain (red) in C. D: Model for the formation of the smooth muscle layers in the gut: The circular layer (c) forms first and gives rise to cells forming the longitudinal layer (l).
eGFP was broadly expressed in acinar and islet cells in neonatal and adult “green mice” and served as a robust label of cellular origin in eGFP ++ 0 mouse chimeras. In adult chimeras, the exocrine pancreas consisted of large acinar patches defined by sharp borders indicating an extensive coherent expansion of acinar clones (Fig. 5A). 3-dimensional reconstructions of huge eGFP-positive patches revealed the existence of surrounding smaller patches (<50 acini, e.g. Fig. 5B, white arrow), however there was hardly any dispersed acinar tissue at the patch borders indicating minimal cell mingling of acinar clones.

In the neonatal pancreas, a small proportion of acini were polyclonal (not shown), but only monophenotypic acini were found in the adult pancreas (Fig. 5A). We suggest that progressive branching during pancreas development gives rise to several oligoclonal protrusions that generate mixed (GFP + 0) and unicoloured GFP or unlabelled acini.

Subsequent growth of the pancreatic lobes with minimal intermingling will lead to an expansion of the monophenotypic areas, which in turn produce monophenotypic acini that need not be monoclonal sensu strictu (Fig. 5D). As in myocard, there was no indication of a secondary contribution to acinar tissue by bloodborne progenitors: large unlabelled acinar regions in low eGFP chimeras were devoid of interspersed small eGFP positive patches.

In addition we found that islets of neonatal and adult chimeras were composed of eGFP and unlabeled cells and concluded that islets of Langerhans were oligoclonal (Fig. 5C, C').

Is labelling really harmless?
What about developmental neutrality? With naturally occurring alloforms of enzymes and other proteins there may be no problem. But with randomly integrated transgenes (as in the case of “green mice”) we do not know which genomic region is affected by the insertion. In fact, green mice seem to be less robust in a colony than controls. Increased copy number of the transgene and high concentrations of its product might reduce the vigor of labelled as compared to unlabelled cells. Acinar cells of eGFP mice were crammed with enlarged vesicles that

Figure 5: Migration, expansion and setting up territorial borders in the pancreas.
Embryo chimeras tell the history.
A, B: Acinar tissue in an adult eGFP ++ 0 mouse chimera. Acini (surrounded by laminin, red), are monophenotypic, i.e. totally eGFP negative or eGFP positive. EGFP negative and positive patches have sharp boundaries. B: 3D reconstruction of eGFP patches in an unbalanced eGFP chimera: A huge and a small (white arrow) acinar patch are visible. Note that the shown patch is not complete and has been cut off at the blunt ends to the left and to the right. C, C': Chimeric islet of Langerhans in an eGFP ++ 0 mouse chimera (i) surrounded by eGFP negative acinar cells. C', enlargement of C, C, C': Overlay of eGFP fluorescence and Hoechst stain. i, Islet of Langerhans; b, blood vessel.
D: Model of pancreatic histogenesis and clonal expansion. Combined spatial and temporal section of the growing dorsal chimeric pancreatic bud, with a potential distribution of unlabeled and eGFP (green) pancreatic precursors.

We hypothesize that protrusions in the early pancreatic anlage originate from a low number of founders and that the resulting clones expand coherently to generate large monophenotypic acinar patches. During this process, these monophenotypic regions (dotted lines) can produce monophenotypic (GFP or 0 = unlabelled) acini, but also mixed (GFP + 0) acini at patch borders. However, progressive growth of monophenotypic protrusions will by far outnumber the mixed acini. As islets randomly assemble from endocrine progenitors (red) and immigrate into the acinar tissue, they are oligoclonal (blue contours) and their composition is independent from that of their acinar neighbourhood. (Eberhard and Jockusch, 2010)
had accumulated GFP (Heimann, Jockusch & Eberhard unpublished); still, no effect on the proliferation or differentiation of these cells was observed. When the transgene, due to a knock-in, has inactivated a gene, as in the case of nLacZ replacing the desmin gene, a haploinsufficiency might be expected. In our nLacZ labelled mice no such effect was observed.

Conclusions

In skeletal and cardiac muscle and in pancreas, clonal expansion during development leads to the appearance of macroscopic patches (oligoclones of like phenotype) arguing against a random mixing of cells during embryonic and postnatal development. In muscle, limited intermingling occurs at the borders of polyclones, in pancreas extremely sharp patch-borders in the acinar tissue persist into adulthood, comparable to the territorial borders between tribes. Under non-pathological conditions (which include the tetraparental status of a mouse chimera), there was no indication a secondary immigration of blood-borne stem cells and their integration in the organ systems studied. On the other hand, a surprising migratory activity of myogenic was observed after muscle transplantation, even across the borders of adjacent muscles, probably (like in human populations) in response to traumatic experiences.

Acknowledgements

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Glossary

Expression of label. For convenience, the term “expression of a protein” will be used to describe its presence, although in its original meaning the term refers to a gene.

Embryo chimeras in mice: These usually originate from the aggregation of two 8-cell stage preimplantation embryos. Their designation is e.g. GFP +/− 0, when one partner is labelled with GFP, the other unlabelled, or GFP +/− nLacZ in the case of complementary label of the two embryos.

Alloform, of enzymes or other proteins. By this we mean natural genetic variants, often involving one amino acid residue, which does not affect function but allows for a physical distinction, e.g. by electrophoresis if a charged residue is involved.

References


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The sequencing of genomes and elucidation of major signalling pathways in model organisms have paved the way towards a mechanistic understanding of control mechanisms that govern organogenesis, differentiation and regeneration of tissues and elucidated pathomechanisms underlying human disease. Without any doubt, understanding the cell biological basis of disease will be a prerequisite for the development and successful application of rational therapies. Owing to its large size, the abundance of stem cells and its accessibility, the skin is ideally suited to identify protein function with relevance for many other organs due to its wonderful genotype-phenotype correlation. In addition to fundamental concepts in immunology, the function of many cytoskeletal and cell adhesion molecules was for the first time identified by pathogenic mutations manifesting in the skin, giving rise to human disorders including epidermolysis bullosa, pemphigus and atopic disease. Given that many of the corresponding genes are organised in families, elucidation of pathomechanisms in skin and its major cell, the keratinocyte, served as paradigms towards other disorders. The identification of the p63 network controlling keratinocyte proliferation and differentiation remained a prerequisite for the successful clinical application of autologous keratinocyte stem cell transplantation following burns, chronic lesions or genetic disorders. The accessibility of the skin has most recently led to first treatments of genetic disorders including epidermolysis bullosa using stem cell approaches and RNAi. Despite the remarkable progress numerous questions regarding cell biology, biophysics and biochemistry of the skin remain open, limiting the development of rational therapies. By now, it is evident that pathomechanisms underlying even monogenic skin diseases, are much more complex than anticipated. Untangling this complexity at the molecular level and its transformation into therapies will undoubtedly depend on an interdisciplinary approach of cell biology, biophysics, genetics, immunology and molecular medicine. The first International meeting „Molecular concepts in epithelial differentiation, pathogenesis and repair“ (http://www.molcedpare.de) brings together internationally recognized colleagues and junior scientists with a major interest in the above topic. From November 7-10, ~30 invited speakers and ~15 junior scientists will discuss major topics in 6 sessions, poster meetings and during a social programme, allowing ample time for informal discussion and initiating scientific collaborations.
New frontiers in studying pseudouridine formation in RNA
Ute Kothe

Pseudouridines
It is more than 50 years ago that a fifth nucleotide has been discovered to be part of RNA. This fifth nucleotide, as it is still sometimes called, is the most abundant, post-transcriptionally modified nucleotide in cellular RNA called pseudouridine. As the name implies, pseudouridines are modified uridines which differ from their parent nucleotide by having a unique C-C glycosidic bond instead of the canonical N-C glycosidic bond (Fig. 1). This modified nucleotide retains the ability to base-pair with adenine; however the additional imino group in the base allows for further hydrogen bonds to form, in particular bridging a water molecule between the base and the preceding phosphodiester bond. As a result, pseudouridines are thought to rigidify RNA in particular by improving base stacking, but roles for the specific function of the modification in non-coding RNA such as ribosomal and spliceosomal RNAs have also been discussed. As pseudouridine has been known for half a century, this raises the question: why are we still interested in this seemingly small adjustment to cellular RNA? There are two main answers to this question which will be both addressed in this review. First, only the last years have allowed us to gain a detailed understanding of pseudouridine syntheses which has in turn raised several new questions. And second, we are only beginning to identify the importance of pseudouridine formation for the cell and its potential applications.

Stand-alone Pseudouridine synthases
Each organism contains several pseudouridine synthases which are responsible for the site-specific modification of many different RNA target sites. To date, very comprehensive knowledge has been accumulated on the different bacterial pseudouridine synthases, their respective target sites and also their crystal structures. This information reveals that, despite significant sequence diversity, all pseudouridine synthases share a common fold in their catalytic domain which is characterized by a central 8-stranded mixed β-sheet that is surrounded by several loops and α-helices (Fig. 2A and B). In one instance (TruD), this catalytic domain is interestingly formed by a circular permutation in its primary sequence. Based on sequence and structure comparison, pseudouridine synthases are classified into six families (Table 1). Representatives of five families are found in all domains of life and are named according to a bacterial representative whereas the sixth family is represented so far by the single enzyme Pusso, present in many archaea and eukaryotes. The active site of pseudouridine synthases is located in a cleft in the middle of the catalytic domain where the universally conserved aspartate residue is found; this so called catalytic aspartate residue is essential for pseudouridine formation. In addition, the active site contains an aromatic residue, usually a tyrosine, that stacks with the target uridine and might play a role as a general base in a late step of the reaction chemistry, as well as a conserved positive arginine residue, that might stabilize or activate the catalytic aspartate residue (Fig. 2A). Aside from the catalytic domain, pseudouridine synthases differ significantly from each other in particular with respect to additional domains which are believed to contribute to specific RNA binding. For example, pseudouridine synthases of the TruB family are characterized by an additional C-terminal PUA domain (found in pseudouridine synthases and archaeosome-transglycosylases) that is believed to bind to the tRNA acceptor stem and possibly also the 3’CCA end of tRNA or the 3’ACA end of H/ACA guide RNA (vide infra). Enzymes of the RsuA family and some members of the RluA family have an N-terminal 54-domain resembling the ribosomal S4 protein and may have C-terminal extensions. The TruD protein harbors a unique domain which is inserted into the catalytic domain. And lastly, the TruA enzyme has been found to operate as a homodimer where each subunit contains an active site and tRNA is bound across both subunits. Stand-alone pseudouridine synthases recognize their target sites through a variety of molecular mechanisms and with different degrees of specificity. Escherichia coli pseudouridine synthases are responsible for generating pseudouridines at seven positions in specific tRNAs as well as eleven pseudouridines in 16S and 23S ribosomal RNA (rRNA). RluA is the only pseudouridine synthase that is capable of modifying both tRNA and rRNA, but other enzymes also target several different sites within tRNA (TruA) or rRNA (RluC, RluD) (Table 1). The specificity of RNA
recognition by pseudouridine synthases can either be achieved by identifying conserved sequence elements within the RNA or by detecting a particular RNA conformation. Interestingly, all crystal structures of pseudouridine synthases in complex with RNA, so far, reveal different mechanisms of recognizing RNA conformations, such as recognition of: (1) the native RNA conformation (TruB), (2) a distorted RNA structure which is induced upon binding the RNA by the protein (RluA and RluF) or even (3) the flexibility of RNA structure allowing it to adopt different conformations (TruA). In all cases, the pseudouridine synthases have to gain access to the target uracil base in order to modify it, and therefore this base is typically flipped out of the RNA structure and into the active site of the modification enzyme. Along with the target uracil, up to two additional bases might be flipped out and buried in binding pockets of the enzyme. Taken together, these mechanisms of target site recognition ensure that pseudouridines are only found in a small number of defined positions in cellular RNA.

While the study of eukaryotic pseudouridine synthases is lagging behind the investigation of their bacterial counterparts, a few interesting points can be noted. So far, eleven pseudouridine synthases in eukaryotes (mostly in Saccharomyces cerevisiae) have been characterized, and their target sites have been identified (Table 1). Strikingly, pseudouridine synthases are not universally conserved, and not all bacterial enzymes have orthologues in eukaryotes. Most notably, no eukaryotic enzyme of the RsuA family has been identified so far. Furthermore, upon comparing bacterial and eukaryotic pseudouridine synthases, it becomes evident that the latter enzymes are mainly responsible for modifying tRNA, sometimes small nuclear RNA (snRNA) and only in one case 5S rRNA and mitochondrial 21S rRNA. This is in clear contrast to the bacterial enzymes which often target ribosomal RNA, and reveals the evolution of pseudouridine synthases to recognize very different and new target sites in the eukaryotic kingdom.

H/ACA small nucleolar ribonucleoproteins and pseudouridine synthases

The most interesting and only essential eukaryotic pseudouridine synthase is the enzyme Cbf5, which belongs to the TruB family, and is also found in archaea. This is the only pseudouridine synthase that does not act alone, but functions in complex with three other proteins, Nop10, Gar1, and Nhp2, as well as with an H/ACA guide RNA, forming an H/ACA small nucleolar ribonucleoprotein (snoRNP) complex (Fig. 2B and C). The clear advantage of these H/ACA snoRNPs over stand-alone pseudouridine synthases is the greatly expanded diversity in recognizing target RNA sites. By applying a “division of labor” approach, the Cbf5 enzyme is
Table 1. Pseudouridine synthases and their target sites in bacteria and eukaryotes.

<table>
<thead>
<tr>
<th>Family</th>
<th>Prokaryotic Enzyme</th>
<th>Target site (E. coli)</th>
<th>Eukaryotic enzyme</th>
<th>Target site (S. cerevisiae)</th>
</tr>
</thead>
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<tr>
<td>TruA</td>
<td>Pus1&lt;sup&gt;60&lt;/sup&gt;</td>
<td>tRNA U77/28/34/36</td>
<td>tRNA U27/28</td>
<td>U2snRNA U26/27/28/34/36/65/67</td>
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<tr>
<td></td>
<td>TruA&lt;sup&gt;60&lt;/sup&gt;</td>
<td>tRNA U38/39/40</td>
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<tr>
<td>TruB</td>
<td>TruB&lt;sup&gt;64&lt;/sup&gt;</td>
<td>tRNA U55</td>
<td>Pus4&lt;sup&gt;65&lt;/sup&gt;</td>
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<td>many – with help of guide RNA</td>
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<tr>
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<td>165 rRNA U516</td>
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<td>tRNA U13</td>
<td>Pus7&lt;sup&gt;75&lt;/sup&gt;</td>
<td>U2snRNA U35, tRNA U13, pre-tRNA&lt;sup&gt;76&lt;/sup&gt; U35, 5S rRNA U50</td>
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<td>none</td>
<td></td>
<td></td>
<td>tRNA U54/55 (archaea)</td>
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</table>

* Pus10 is not found in S. cerevisiae, but in humans and other eukaryotes; the target site has only been characterized in archaea.

merely responsible for catalyzing the modification<sup>44</sup> while it is the H/ACA guide RNA that specifically recruits target RNA into the complex for modification.<sup>25</sup> There are many different H/ACA guide RNAs (more than 100 in humans) that allow the H/ACA snoRNPs to modify a large number of uridines in a site-specific manner. Interestingly, for several H/ACA guide RNAs no target sites have been identified so far. Eukaryotic H/ACA guide RNAs are characterized by having two stem-loop structures that are separated by a characteristic ANANNA Hinge region and by containing a 3'ACA sequence giving rise to their naming as H/ACA guide RNAs (Fig. 2B). Within each stem-loop structure is an unpaired region called pseudouridylation pocket; both the 5' and the 3' side of the pseudouridylation pocket base-pair with the target site bulging out the target uridine and a flanking residue thereby allowing the target uridine to bind to the catalytic pocket of Cbf5.<sup>45</sup> The last decade has greatly contributed to our understanding of H/ACA snoRNP structure and function, in particular based on biochemical studies of archael ribonucleoprotein complexes. A number of detailed reviews on the complex biogenesis, function and structure of H/ACA snoRNPs have been published in the last years.<sup>26–28</sup>

Tackling the catalytic mechanism of pseudouridine synthases

Despite the wealth of biochemical information on pseudouridine synthases, the chemical mechanism of pseudouridylation still remains unsolved. As of now, three different chemical mechanisms have been suggested, but for a long time it was even unclear whether all pseudouridine synthases share the same mechanism or whether different enzyme families might utilize different chemistry. Three arguments now support the general opinion that all pseudouridine synthases apply the same chemical strategy. First, the structural studies have revealed a common catalytic domain including a conserved catalytic pocket with an essential aspartate residue even in only distantly related families such as TruD.<sup>7</sup> This finding also indicates that all pseudouridine synthases were probably derived from a common ancestral enzyme. Second, the interaction of pseudouridine synthases with RNA containing the inhibitor 5-fluorouracil has now been clarified. Originally, it seemed as if only some enzymes form covalent adducts with 5-fluorouracil (e.g. TruA)<sup>44</sup> while others are capable of converting this substrate to a 5-fluoro-6-hydroxypseudouridine species (e.g. TruB).<sup>44</sup> However, a careful study by the Mueller group has now revealed that all enzymes seem to equally react with 5-fluorouracil thus eliminating the suggested difference between the enzymes.<sup>29</sup> Lastly, our group has conducted a detailed kinetic study of bacterial pseudouridine synthases representing three different families which demonstrated that pseudouridine synthases are characterized by a uniformly slow catalytic step.<sup>31</sup> The surprisingly identical rate constants for pseudouridine formation can be explained by a common, limiting chemical mechanism.

Pseudouridine formation is a rather complex process as it minimally comprises three chemical steps including the cleavage of the N-C glycosidic bond, the rotation of the base within the enzyme’s active site and the
re-attachment of the base to the ribose by forming the C-C glycosidic bond found only in pseudouridines. As mentioned above, three different catalytic mechanisms have been suggested for pseudouridine synthases which can be distinguished by the role of the catalytic aspartate; in particular, which site of the target uridine is attacked by this aspartate, and by the nature of the intermediates. Interestingly, the first step during pseudouridine formation, glycosidic bond cleavage, is the same as the reaction catalyzed by uracil-DNA-glycosylases which also employ a catalytic aspartate residue.\(^9\) This similarity was the basis for the first proposed catalytic mechanism suggesting a nucleophilic attack by the aspartate onto the C3' of the ribose resulting in an acylal intermediate (Fig. 1).\(^{15}\) Alternatively, it was instead hypothesized that the catalytic aspartate residue could form a covalent bond to C6 of the uracil base creating a Michael adduct.\(^{16}\) A covalent bond between the aspartate and the base could then constitute the axis of rotation for the base to position the C5 next to the C3' of the ribose. Both mechanisms were debated for more than a decade. Most recently however, the Mueller group extended the studies on the exact nature of the 5-fluoro-6-hydroxypseudouridine product and identified two isomeric hydrazide products.\(^{17}\) Importantly, these findings strongly disfavor the mechanism forming a Michael adduct, but are in agreement with a modified mechanism including the suggested acylal intermediate. Alternatively, the authors propose a third catalytic mechanism where the catalytic aspartate abstracts a proton from the C2' position of the ribose creating a glycal intermediate and a free uracil base. Thus, the field is completely open again and further studies are required to verify the catalytic mechanism. Clearly, new experimental approaches are needed to resolve this long-standing question. For example, kinetic isotope studies can reveal whether C2' is closely involved in catalysis; however, such studies are extremely challenging based on the large size of the substrate RNA which has to be specifically labeled with a heavy isotope. Alternatively, it might be possible to trap and isolate reaction intermediates and to characterize their chemical structure, but again this is no easy task. Hence, a half-century after the discovery of pseudouridines, we are still not able to explain how these modifications are formed, but the progress in the past decade gives rise to the hope that this important question might finally be answered in the near future.

Identifying the cellular functions of pseudouridine synthases

Although pseudouridines are the most common post-transcriptional modification found in RNA across all domains of life, our understanding of the importance of pseudouridines and pseudouridine synthases for the cell still remains fragmented. As most pseudouridines are found in ribosomal RNA, in particular at the functional centers of the ribosome such as the peptidyltransferase center and the interface of the large and small subunit, a role of pseudouridines in ribosome bioge-
ness and function would be expected. In the last decade, the Fournier lab in particular has analyzed the impact of deleting specific pseudouridines from ribosomal RNA in yeast. For this purpose, single H/ACA guide RNAs were knocked out or mutated to remove a single or a small number of site-specific pseudouridines that are introduced into rRNA by H/ACA small nucleolar ribonucleoproteins. Most interestingly, the lack of a single pseudouridine usually minimally affects the ribosome or the yeast cell. However, the removal of more than three pseudouridines from a single area of the ribosome such as the peptidyltransferase center, helix 69 in 25S rRNA at the interface with the small ribosomal subunit, the A-site finger of the large ribosomal subunit, or the decoding center, all lead to synergistic effects of varying degrees on cell growth, translational activity and ribosome formation. Interestingly, removal of just a single pseudouridine in many of the mentioned functional regions of the ribosome can also affect the accuracy of translation.

Taken together, these findings suggest that many pseudouridines contribute together to the function and formation of the ribosome thereby enhancing cellular fitness, even though the role of individual pseudouridines might be minimal. In accordance with these studies in yeast, three pseudouridine modifications in helix 69 of the bacterial ribosome, that are introduced by the pseudouridine synthase RluD, have also been shown to be important for ribosome function as they influence the interaction of the ribosome with release factor 2 (which is not a homologue of eukaryotic release factors). Several pseudouridines are also found in spliceosomal RNA, in particular U2 snRNA, where they seem to contribute to spliceosomal function. Almost all uridines in the branch site recognition sequence of U2 snRNA are modified to pseudouridines. Conversion of these sites to 5-fluorouridines, that cannot be changed to pseudouridines, inhibited splicing in a Xenopus oocyte reconstitution system. Similarly, the lack of several pseudouridines in the 5’ region of U2 snRNA results in cumulative splicing defects, in particular the formation of the spliceosomal E complex. The most important spliceosomal pseudouridine is found at position 35 in the branch site recognition sequence of yeast U2 snRNA. Deletion of the pseudouridine synthase Pus7p that generates this modification leads to growth defects under stress conditions including pre-mRNA accumulation. U2 pseudouridine 35 has a structural effect on branch site recognition sequence and helps to position the branch site adenosine for nucleophilic attack of the 5’ splice site. In summary, at least a subset of pseudouridines seem to be important for the function of the ribosome and the spliceosome while other pseudouridines might be less critical for the function of these ribonucleoprotein machines, and may act in a synergistic structural context.

In accordance with the findings that single pseudouridines are dispensable in the cell, most pseudouridine synthases are not essential enzymes with the notable exception of Cbf5, that is responsible for the modification of large numbers of uridines by H/ACA small nucleolar ribonucleoproteins. Surprisingly however, yeast cells are viable, but grow poorly, when expressing only an inactive Cbf5 variant, where the catalytic aspartate has been mutated such that in vivo pseudouridylation is abolished. This raises the question whether a function of Cbf5 other than its pseudouridylation activity is essential for the cell. Presumably, this essential function of Cbf5 resides in its contribution to pre-rRNA processing, in complex with the snR30 RNA, the only essential H/ACA box RNA in yeast, Cbf5 somehow is required for cleavage of the pre-rRNA transcript while the snR30 H/ACA box RNA seems not to direct pseudouridylation events. All E.coli stand-alone pseudouridine synthases can be knocked out without detectable effects on cell viability. Interestingly, a knock-out strain of E. coli lacking TruB (a homologue of Cbf5) grows well on its own but is out-competed by wild-type strains, indicating a contribution of TruB to the fitness of E. coli cells. The same finding holds true for an E.coli RluA knock-out strain. Strikingly, this phenotype of the TruB knock-out strain can be reversed by expression of a catalytically inactive TruB mutant, further suggesting that an uncharacterized function of this pseudouridine synthase (other than RNA modification) is critical for cellular fitness. This “other” function of TruB might be related to thermal stress tolerance since both a TruB knock-out strain as well as a strain expressing inactive TruB48C display sensitivity to a temperature shift from 37 to 50°C. What function of pseudouridine synthases in addition to pseudouridine formation can be important to the cell? An unambiguous answer is still lacking, but it has been hypothesized that these RNA modification enzymes might also act as RNA chaperones that contribute to the folding of their target RNA independent of the chemical modification. For example, many pseudouridine synthases need to gain access to the modification site by changing the structure of their substrate RNA and by flipping nucleobases into their active site (vide supra). This interaction with pseudouridine synthases might help to unfold non-native structures of the RNA and to provide the RNA with a second chance of folding to reach its native state upon dissociation from the pseudouridine synthases. Such an RNA chaperone activity of pseudouridine synthases might not be required under normal growth conditions, but only under certain stress conditions again highlighting that these enzymes contribute to the general fitness of cells without being essential. Similar to this suggestion, it has been demonstrated that knock-out of the E.coli pseudouridine synthase RluC, that targets the ribosomal peptidyltransferase

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Using pseudouridine formation to regulate gene expression

A very new and exciting question in the field of RNA modification is whether pseudouridines are used by the cell or could be used as artificial tools in order to regulate gene expression. The last few years have in fact revealed astonishing findings supporting both suggestions. First, the potential of pseudouridines to contribute to gene regulation in the cell will be reviewed. The best example of such regulation is the inducible pseudouridylation of U2 snRNA which influences pre-mRNA splicing. As described above, several pseudouridines, in particular pseudouridine 35 in U2 snRNA, are important for splicing. In addition to these constitutive pseudouridines, the Yu group has identified two inducible pseudouridines in yeast U2 snRNA that are introduced by the stand-alone pseudouridine synthase Pus7p and by H/ACA small nucleolar ribonucleoproteins containing the guide RNA snR81, respectively. These pseudouridines are only found under nutrient deprivation or upon heat shock. At least one of these pseudouridines (Ψ93 introduced by snR81 snoRNPs) influences pre-mRNA splicing.

The second example of cellular regulation of gene expression is the pseudouridylation of steroid receptor RNA activator. Here, the stand-alone pseudouridine synthase Pus1 modifies steroid receptor RNA activator and acts thereby as co-activator for nuclear receptors such as the retinoic acid receptor by forming a steroid-independent trimeric complex of Pus1, steroid receptor RNA activator and retinoic acid receptor. The physiological significance of this co-activation is currently unclear.

Finally, recent studies have revealed that the pseudouridylation machinery is linked to mi-croRNAs that are major regulators of gene expression. Strikingly, H/ACA small nucleolar RNAs, which normally act as part of H/ACA small nucleolar ribonucleoproteins, are sometimes also precursors of microRNAs. Hence, some of these RNAs have a dual functionality and can act both as guide RNAs directing pseudouridylation as well as precursors for well-established microRNAs which regulate gene expression. The evolutionary link between snoRNAs and microRNAs is an interesting question that warrants further investigation.

Beyond the described cases of regulation of gene expression by the pseudouridylation machinery, there may be many more mechanisms. For example, C/D box small nucleolar guide RNAs target post-transcriptional 2'-O-ribose methylation of RNA and therefore act similarly to H/ACA snoRNAs as guide RNAs. In addition, they have also been shown to have other functions, such as interacting with pre-mRNAs to influence alternative splicing patterns. As there are also numerous H/ACA snoRNAs for which no target pseudouridine site has been identified yet, it might be possible that similar regulatory mechanisms exist for H/ACA guide RNAs. So far, no pseudouridines have been detected in cellular mRNA, but this can be attributed to the low abundance of some of these RNAs and the technical difficulty of detecting pseudouridines. As these modified nucleotides base-pair to adenine like uridines and also have the same molecular mass, high-throughput detection of pseudouridines by reverse-transcriptase based sequencing strategies or mass spectrometry of cellular RNA is currently not possible.

With our increasing understanding of pseudouridine formation, it now becomes possible to use this knowledge as a tool to manipulate cells. First, it has been recognized that incorporation of pseudouridines into mRNA by in vitro transcription renders such mRNAs more stable and thus translationally

**Figure 3: Regulation of gene expression by pseudouridines and H/ACA box RNAs.** Several reports have now revealed how pseudouridine formation in snRNA, mRNA and steroid receptor RNA activator can influence gene expression (see main text for details). Furthermore, H/ACA box RNAs can be precursors of microRNAs independent of their function in directing pseudouridylation. Other mechanisms of regulating gene expression by pseudouridines or the pseudouridylation machinery can be envisioned and might be discovered in the future (indicated by question marks).

\[ \text{pseudouridines protect mRNA against immune recognition} \]

\[ \text{pseudouridines in stop codons induce translational read-through} \]

\[ \text{re-coding of sense codons by pseudouridines in mRNA??} \]

\[ \text{inducible pseudouridines in siRNA influence splicing} \]

\[ \text{H/ACA box RNAs} \]

\[ \text{binding to pre-mRNAs influencing splicing??} \]
active upon delivery to cells. This effect stems from the inability of pseudouridine-containing mRNAs to activate 2’-3’-oligoadenylyl synthetase and subsequently RNase L. Furthermore, pseudouridine-containing mRNAs are resistant to degradation by RNase L. Thus, incorporating pseudouridines into mRNAs constitutes a mechanism to overcome immune recognition and in the future may allow using such mRNAs in gene replacement and vaccination.

Second and very impressively, the Yu group recently demonstrated that directed pseudouridylation of stop codons in mRNA results in translational read-through, thus opening the exciting possibility of using site-directed pseudouridylation for targeting aberrant mRNAs produced in genetic diseases that are based on the occurrence of premature stop codons. The authors made use of the conserved secondary structure of HACA guide RNAs to design novel guide RNAs that base-pair to mRNA nucleotides flanking the target uridine residue at the first position of the stop codon, thereby specifying this residue for modification by HACA small nucleolar ribonucleoproteins. For all three stop codons, this strategy resulted in significant read-through of pre-mature stop codons both during in vitro translation as well as in S. cerevisiae; this effect was enhanced if the nonsense-mediated decay pathway was inactivated. Hence, disease-specific guide RNAs might be designed in the future and delivered to affected cells in order to use intrinsic HACA small nucleolar ribonucleoproteins to modify mutated mRNA and to thereby overcome premature termination in these patients. Surprisingly, the pseudouridine-containing codons are read by very specific tRNAs resulting in the incorporation of not more than two different amino acids at the corresponding position in the encoded polypeptides. This altered decoding potential by the ribosome was entirely unexpected and may allow expansion of the genetic code. Future studies will reveal whether the modification of sense codons also likely leads to the incorporation of specific, different amino acids and would therefore open even more possibilities for developing alternatives and additions to the genetic code.

Conclusions

Although pseudouridines have been discovered more than half a century ago, the last decade has revealed remarkable features of pseudouridines and pseudouridine synthases thereby revolutionizing our understanding of this post-transcriptional RNA modification. We are only beginning to comprehend the molecular mechanism of target recognition and chemical conversion of uridines by pseudouridine synthases which are catalyzing a remarkably complex chemical reaction. It seems that the foundation has now been laid to address these questions in the future. Even more complex is the biological function of pseudouridines which have synergistic effects on ribosome and spliceosome function and contribute the cellular fitness under certain stress conditions. Very surprisingly, the actual chemical conversion of uridines to pseudouridines might have secondary importance to the presence of the pseudouridines synthases themselves. These enzymes may have additional cellular functions such as acting as RNA chaperones to enhance the folding of cellular RNAs. This intriguing hypothesis certainly needs further investigation to clearly identify the cellular role of pseudouridine synthases. Lastly, a number of very recent studies suggest that pseudouridine formation may be regulated in certain cases in the cell and may thus contribute to the regulation of gene expression under stress conditions or in specific tissues. Also, HACA guide RNAs can have dual functionality as they can be precursors of microRNAs, but it is not clear under which conditions a guide RNA is further processed to a microRNA. A better understanding of such regulatory functions may significantly enhance our knowledge of pseudouridine function in health and disease in the future. As a first glimpse of how to use pseudouridine formation to influence cells, an amazing new possibility of changing the genetic code by introducing pseudouridines into mRNAs has recently emerged, which has large potential in the treatment of inherited diseases as well as in engineering novel polypeptides using an expanded genetic code. After more than 50 years of research, the field of pseudouridine research seems more exciting and promising than ever before.

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Ute Kothe studied Biochemistry at the universities in Regensburg, Bochum and Witten. From 2002 to 2006 she completed her Ph.D. studies in physical biochemistry under the supervision of Dr. Marina Rodnina at the University of Witten/Herdecke where she investigated the kinetics and accuracy of bacterial protein synthesis. Subsequently, Ute Kothe accepted a position as Assistant Professor at the University of Lethbridge in Alberta, Canada in 2006 where she is now tenured. Her research group investigates the early steps of ribosome biogenesis with a special focus on RNA modification and folding. By using model systems ranging from bacteria, to archaea and yeast as well as a combination of molecular biology, genetics, biochemistry and biophysics, in particular kinetics and fluorescence, Ute Kothe’s research currently addresses the molecular mechanisms underlying RNA-protein interactions during modification and RNA folding. Ultimately, these investigations will not only increase our fundamental knowledge on how the cells builds complex ribonucleoproteins, but will also aim at identifying novel targets in the treatment of cancer, other proliferative diseases and inherited diseases which all affect ribosome biogenesis.
Unraveling functions of the keratin multiprotein family using a genome strategy

Kristin Seltmann, Wera Roth, Cornelia Kröger, Preethi Vijayaraj, Fanny Loschke & Thomas M. Magin

Introduction
Higher organisms are characterized by a bewildering number of cell types which participate in tissue and organ formation. The increase in cell types found in vertebrates compared to lower organisms is not fully matched by an increase in the number of protein-coding genes. During evolution, a number of genes have undergone duplications, resulting in gene families encoding proteins with cell type-specific functions. Among these gene families are those encoding the keratin multiprotein family. Encapsulating 54 genes in the human and the mouse, which are subdivided into the type I and type II keratin families with 28 and 26 members, respectively, the keratin family is among the 100 largest gene families in mammalian species (Venter et al., 2001; Lander et al., 2001). Keratins form the intermediate filament (IF) cytoskeleton in all epithelia cells and act as supramolecular scaffolds by interacting with cell-matrix and cell-cell contacts (Fuchs and Cleveland, 1998; Simpson et al., 2011). Thereby, they contribute significantly to epithelial tissue functions including metabolite exchange, communication, protection against dehydration, infections, pharmacological and physical stress. Most epithelia express between 6-10 different keratins as pairs of distinct type I and type II proteins (Schweizer et al., 2006; Magin et al., 2007). In the basal epidermis, the keratin pair K5/K14 is expressed, which upon terminal differentiation becomes sequentially replaced by Ks/Ks0 in suprabasal keratinocytes where they support cornified envelope formation (Coulombe and Wong, 2004). The ad- aptive nature of keratin expression becomes apparent during wound healing and closure, characterized by profound and transient changes in keratin organization and isotype expression. In stratified epithelia, migration into the wound starts from suprabasal epidermal layers (Coulombe, 1997). During re-epithelialization, expression of K6, K16 and K17 is induced, accompanied by the down-regulation of differentiation-specific K1 and K10. This correlates with alterations in cell morphology and migratory properties (Paladini et al., 1996; Patel et al., 2006). In addition, it was shown that alterations in K6, K16 and K17 expression also influence wound healing in vivo and keratinocyte migration ex vivo (Wong and Coulombe, 2003; Mazzalupo et al., 2003; Wawersik and Coulombe, 2000). Like in many other multigene families, the major question to be answered remains “do keratins have unique, isotype-specific functions and if so, what are these?” Classic genetic gain and loss-of-function approaches have not fully resolved this issue, owing to keratin redundancy (Magin, 2004; Vijayaraj et al., 2007; Vijayaraj et al., 2009). We have recently devised a “delete and replace” strategy to overcome this limitation. It is based on our observation that keratin genes are grouped in 2 clusters on chromosomes 11 and 15 in the mouse, spanning ~0.68 MB for the type II and 1.2 MB for the type I genes, without any other known protein-coding genes located in these clusters (Schweizer et al., 2006; Hesse et al., 2001). Having recognized this, we set out to delete the entire type I and type II gene clusters separately in murine ES cells, using a cre/loxP-mediated deletion strategy (Vijayaraj et al., 2009). We reasoned that it should be feasible to generate mice carrying deletions of the entire type I or type II clusters in a spatio-temporal fashion upon mating to appropriate cre-expressing mouse lines. These should be accessible to phenotypic analysis without compensatory keratin expression and give rise to novel cell lines devoid of all keratins. Ultimately, it should be possible to re-express single keratin pairs in cell lines and mice to identify true keratin isotype functions.

Figure 1: Structural organization of hemidesmosomes. Hemidesmosomes connect the intracellular keratin cytoskeleton (K5/K14) with the extracellular matrix. The participating transmembrane proteins BP180, CD 151 and α6β4-integrin directly interact with the extracellular ligand laminin-332. In addition, they stay in contact with keratin-associating proteins plectin and BP230.
Research strategy and first results
Provided our strategy would work, what could be the outcomes? In view of the fundamental importance of keratins for the maintenance of epithelial integrity, in particular in the epidermis (Gu and Coulombe, 2007) and knowing that keratin expression commences in the 2 cell stage (Lu et al., 2005), two major concerns were raised by colleagues, grant reviewers and by ourselves: 1) Can we learn a new lesson? 2) Would epithelia form in the absence of keratin IF? Given that type I or type II keratins are unable to form IF on their own, one of the additional questions was “will type I and II deletion in the same tissue lead to the same phenotypic changes”? Another concern was whether the deletion of keratins would be compensated by upregulation of other keratin IF proteins, e.g. vimentin. For technical reasons, deletion of the type II gene cluster was performed first. In contrast to prediction, embryonic epithelia formed in the absence of keratins without any sign of cytolyis or loss of apical polarity, thereby challenging findings from single keratin gene knockouts (Magin et al., 2007). Surprisingly, all keratin-null embryos died from severe growth retardation at embryonic day 9.5. We found without keratins, GLUT1 and -3 were mislocalized from the apical plasma membrane in embryonic epithelia, which subsequently activated the energy sensor adenosine monophosphate kinase (AMPK) (Vijayaraj et al., 2009). Analysis of the mammalian target of rapamycin (mTOR) pathway revealed that AMPK induction activated Raptor and repressed protein biosynthesis through mTORC1's downstream targets S6 kinase and 4E-binding protein 1 (Vijayaraj et al., 2009; Vijayaraj et al., 2010). Further, we found that keratins are necessary to maintain adhesion between endodermal and mesodermal cell layers of the yolk sac. As a consequence, keratin-deficient embryos suffered from reduced yolk sac hematoipoiesis and vasculogenesis. Pathway analysis revealed a reduction of the hedgehog target Foxf1 in yolk sac mesoderm of keratin-/- embryos, and subsequent reduction of BMP-4 and P38 MAPK. This indicated for first time that keratins are necessary for the differentiation of a non-epithelial cell lineage through a combination of mechanical and signaling mechanisms.

Since then, we extended our strategy to other epithelia and included deletion of the type I cluster in the analysis. Mating Krt type I-floxed mice with HPRT-cre mice should result in the deletion of all type I keratins early in development except Krt18. Together with Kt8, Krt18 is located in the type II cluster and these two proteins represent the first keratin pair during embryogenesis and the predominant pair in all simple epithelia (Omary et al., 2009). Thus, this experiment addresses the questions what is the function of K19, K20 and K23 in specialized epithelial cell types, or “are Krt8 and Krt18 sufficient”? Provided that Krt8 and Krt18 suffice, type I keratin deletion permits analysis of epidermal development without keratins, which commences beyond embryonic day 9.5 when Krt type II deleted embryos die. – These experiments are currently underway.

To address keratin function at the cellular and molecular level and to test the potential of our “delete and replace” approach, we isolated keratinocytes from mice heterozygous for the type II keratin deletion and removed the second allele ex vivo (Loschke et al., in prep.). This resulted in 3 distinct keratinocyte cell lines, one that lacks all keratins, one that re-expresses the keratin pair Krt5 and Krt14 and a wildtype counterpart. In the remaining text, we focus on one aspect that has uncovered the involvement of keratins in cell matrix adhesion, in particular their role in hemidesmosome maintenance.

Cell-matrix contacts in skin
Cell-matrix adhesion is crucial for a variety of biological processes in skin, including skin development, wound healing, inflammation and malignant progression (Ridley et al., 2003). In the skin, matrix adhesion is main-

Figure 2: Characterization of keratin-free keratinocytes. Absence of keratins in keratin-free cells and re-expression of keratin 5 in rescued cells were proved by western blotting.

keratin 5 plectin β4 integrin merge

WT

KO

Rescue
tained by actin-associated focal adhesions and keratin-dependent hemidesmosomes. While the former are well characterized, relatively little is known about mechanisms regulating the interactions of keratins during hemidesmosome formation and maintenance. Disruption of the keratin-hemidesmosome multiprotein complex gives rise to epidermolysis bullosa, a group of severe skin disorders caused by mutations in genes encoding either hemidesmosomal proteins or keratins K5 and K14 (Fine, 2010). The binding of plectin to α6β4 integrin is regarded to be essential for the assembly and stability of hemidesmosomes (de Pereda et al., 2009). Plectin interacts with at least three binding sites to β4 integrin mainly through the N-terminal ABD (actin binding)-domain (Koster et al., 2004). Binding to keratin intermediate filaments involves the C-terminal plakin repeat domain. Owing to its binding sites for actin, intermediate filament proteins and microtubules, plectin qualifies to coordinate the dynamics between hemidesmosomes and focal adhesion dynamics through rearranging the actin and keratin cytoskeletons (Tsuruta et al., 2011; Ozawa et al., 2010; Andra et al., 2003). Hemidesmosomes connect the keratin cytoskeleton (K5/K14) with the extracellular matrix (Jones and Green, 1991). The transmembrane proteins BP180, CD 151 and α6β4 integrin directly interact with the extracellular ligand laminin-332 (Jones et al., 1998; Sterk et al., 2000; Borرادori and Sonnenberg, 1999). In addition, they contact keratin-associating proteins plectin and BP230 (Green et al., 1992) (Figure 1). The interaction of plectin with α6β4 integrin via the ABD-domain and with keratins via the plakin repeat domains on the C-terminal site is necessary for hemidesmosome assembly (Koster et al., 2004).

During migration and adhesion, hemidesmosomes and focal adhesions are continuously remodeled (Geußen and Sonnenberg, 2002; Tsuruta et al., 2003). In this context, serine phosphorylation of β4 integrin leads to a destabilization and relocation of hemidesmosomal proteins into lamellipodia (Litjens et al., 2006).

Keratin filaments maintain the localization of hemidesmosomes

First of all, the absence of all keratins in keratin-free keratinocytes was confirmed by immunofluorescence and western blotting (Figure 2). K5 re-expressing keratinocytes (Rescue) showed a co-localization of K5/K14, forming IF, whereas K6 were absent (not shown). To investigate whether the keratin cytoskeleton effect hemidesmosomes as the major epidermal cell-matrix contact, we analyzed the distribution of the hemidesmosomal proteins plectin and β4 integrin in keratin-free cells. Most remarkably, plectin was completely dissociated from β4-integrin

keratin-free keratinocytes, therefore the hemidesmosomal proteins were no longer clustered like in the wildtype keratinocytes (Figure 3). At the same time, co-localization of β4-integrin and extracellular ligand laminin-332 was not affected in keratin-free cells (not shown). Stable re-expression of K5 and K14 in keratin-free keratinocytes reconstituted the typical localization of plectin to β4-integrin and clustering of hemidesmosomal proteins, demonstrating keratin dependence of the phenotype. Our data provide evidence for a crucial role of keratins in the localization and maintenance of hemidesmosomal proteins, based on the observation that loss of keratins caused an altered distribution of plectin and β4-integrin. In line with the established function of plectin in hemidesmosome assembly and maintenance, our

Figure 3: Altered localization of hemidesmosomes in keratin-free keratinocytes. Immunofluorescence analysis of wildtype (WT), keratin-free (KO) and keratin 5 re-expressing (Rescue) keratinocytes stained against keratin 5, plectin, β4-integrin and laminin-332 to visualize hemidesmosomes structures. In IF analysis of wildtype (1) and keratin 5 re-expressing keratinocytes (3) a characteristic patchy pattern of hemidesmosomal proteins can be seen. Keratin-free cells (2) have a clustered localization of β4-integrin in the cell layer, but no co-localization with plectin. Bar, 10 μm.
data support a novel role of keratins acting through plectin (Koster et al., 2003). Possibly, lack of keratins triggers a conformational change of plectin at its C-terminal IF-binding site which might inhibit the N-terminal binding to β4-integrin (de Pereda et al., 2009).

**Keratins negatively regulate migration**

Given that cell-matrix contacts play an important role in cell motility, we then analyzed if the altered distribution of hemidesmosomes has functional consequences in migration by an established in vitro gap closure assay. Remarkably, keratin-free keratinocytes migrated nearly two-fold faster than wildtype cells, taking ~16 hours to close the gap, whereas wildtype cells required ~28 hours. Consistent with a major role of keratins in restricting migration, K5/K14 re-expressing KO keratinocytes closed the gap in 28h similar to wildtype cells. Thereby, keratin-free cells migrated faster and also displayed an altered directionality compared to wildtype cells. Thereby, faster migration of keratin-free cells coincided with altered distribution and dynamics of hemidesmosomal proteins, consistent with the plectin-dependent clustering of β4-integrin. In fact, keratin-free keratinocytes migrated faster than wildtype cells and showed a reduced directionality during migration. Mechanistically, this could be due to an increased turnover of hemidesmosomes in keratin-free keratinocytes. In support, both plectin and β4-integrin knockout keratinocytes displayed enhanced migration (Raymond et al., 2005; Osmanagic-Myers et al., 2006). Furthermore, α6β4-integrin was shown to stabilize lamellipodia and therefore affects cell motility (Rabinovitch and Mercurio, 1997). Recent data report a role of hemidesmosomes as signaling platforms in which α6β4-integrin signaling is mediated through plectin. Plectin interacts with RACK1, which is linked to the PKCδ signal pathway (Osmanagic-Myers et al., 2006). Furthermore, a role of hemidesmosomes in upstream signaling of the kinases PKB and Erk was shown (Kippenberger et al., 2010). It is conceivable that loss of the keratin cytoskeleton and therefore decreased maintenance of hemidesmosomes also influences intracellular signaling pathways, which affects the migration of keratinocytes. In support of our data, high expression of keratin 16 in cultured skin explants was accompanied by decreased migration (Wawersik and Coulombe, 2000). Here, we demonstrated a requirement of the keratin cytoskeleton in the maintenance and distribution of hemidesmosomes upstream of plectin, which has implications for keratinocyte migration. Our results show that keratins are necessary for the maintenance of intact hemidesmosomes, most likely upstream of plectin. Of note, the single keratin pair K5 and K14 was sufficient for this function. Whether maintenance of hemidesmosomes relies directly or indirectly on keratins requires more detailed investigation.

In summary, our “delete and replace” strategy has provided novel insights into keratin functions, both during embryogenesis and now in hemidesmosome maintenance. The existing cell culture models now enable us to test a number of hypotheses regarding general and isoform-specific keratin functions relevant for epithelial homeostasis and pathogenesis. An attractive hypothesis to be tested is whether the keratin-hemidesmosome complex has a role in mechanotransduction.

**Acknowledgments**

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**Abbreviations**

BP: bullous pemphigoid  
EBS: epidermolysis bullosa simplex  
HD: hemidesmosome  
K: keratin

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Role of biomechanics in the regulation of transendothelial migration and invasion of cancer cells
Claudia Tanja Mierke

Introduction
The migration and invasion of malignant cells into connective tissue microenvironment has long been focused on the analysis of genetic alterations affecting cell adhesion molecules, focal adhesion proteins and their signaling pathways during the progression of cancer disease. Numerous protein mutants have been investigated that showed for example altered cell motility and tumorigenicity. During the last years, biomechanical properties of cancer cells have become the novel focus of recent research studies (1-6). As the surrounding microenvironmental tissue of neoplasms and tumors is altered in terms of mechanical properties compared to “normal” tissue of healthy species, it has been suggested that the biomechanical properties of cancer cells such as the stiffness (the inverse of the compliance) are altered by the microenvironment (Figure 1). Hence, the analysis of biomechanical properties of cancer cells and their microenvironment has become a main focus of biophysical research. In particular, for example the non-receptor protein-tyrosine kinase focal adhesion kinase (FAK) and the focal adhesion protein vinculin are involved in the regulation of many cellular biomechanical processes such as cell cycle progression, cell survival, and migration of cells. Previously, it has been started to analyze the mechano-coupling or mechano-regulating-function of vinculin or FAK in terms of their role in facilitating the mechanical properties of cells (7-10). Thus, this article discusses how the mechanical properties of (cancer) cells determine cellular invasiveness into extracellular matrices (ECMs) or motility. Finally, this article points out the impact of the regulation of cellular biomechanics, and subsequently how this affects the motility or invasiveness of (cancer) cells.

Biomechanics of cancer cells influence their invasiveness
The mechanical properties of the cancer cells regulate their migration efficiency and speed through connective tissue (Figure 2). The invasiveness of cancer cells seems not to be re-
gulated by a single biomechanical parameter. It is rather a set of biomechanical properties that act together to increase (cancer) cell invasiveness. For example, cellular stiffness influences together with the cytoskeletal remodeling dynamics and the transmission or generation of contractile forces the invasiveness of (cancer) cells into 3D ECMs (2, 5, 8).

**Contractile forces:** Cells were able to adhere to the connective tissue ECM through cell-matrix adhesion molecules. Adhesion sites of cells with mesenchymal origin have been broadly investigated on flat and rigid 2D substrates. All cell-matrix contacts contain integrins as their major transmembrane receptors (11-12), which transmit forces derived from the ECM to the interior of the cell, and, in turn, cytoskeletal generated forces are transmitted to the exterior. In stationary cells, firmly adhered to the ECM, external and internal forces cancel out each other, whereas in motile cells, the force distribution is polarized and shifted towards one direction resulting in cell contraction, extension and translocation (13-14). As forces are applied through adhesion molecules towards the ECM, (cancer) cells continuously respond by exerting reciprocal contractile forces to external forces that are generated within the ECM and surrounding cells (13). Anisotropy of the cell’s adhesive microenvironment controls the intracelluar organization and regulates the polarity of the cell (15), which then determines the motility of cells in 3D-ECMs.

**Cell stiffness (inverse of the compliance):**

Recently, our group reported that stiffer cells migrate at higher numbers and further into 3D-ECMs using magnetic tweezer micro rheology (Figure 3) (8). The difference in stiffness comes from a difference in vinculin gene expression: vinculin expressing cells are stiffer compared to their wildtype counterparts. In addition, even breast cancer cells with a minimal 2-fold increased stiffness migrate at higher degrees into 3D-ECMs (5).

**Cytoskeletal remodeling dynamics:** Cells that invade into 3D ECMs (highly invasive cells) showed a higher migration speed and higher cytoskeletal remodeling dynamics compared to cells that were less invasive cells (5). If a cell is able to restructure its cytoskeleton, when it is appropriate as for amoeboid migration, it is then able to squeeze through the pores of a matrix.

### Biomechanics of the tumor microenvironment regulate cancer cell invasiveness

The cellular microenvironment in tissues differs in composition between the various parts of the body. Due to the stiffness of the ECM, the resulting tissue deformations are almost modest.

**ECM stiffness:** Cancer cells interact with the elastic ECM which provides, besides chemical signals, mechanical signals revealing its physical nature. The mechanical properties of the ECM are important in regulating the behavior of cancer cells. A mechanical parameter is the matrix stiffness and a measure of resistance exhibited by the elastic ECM to deformation. In malignant cancer progression, the biomechanical homeostasis is often deregulated, and cancer cells exhibit increased stiffness compared to healthy tissues (16). Thus matrix stiffness has been implicated in tumorigenesis and investigation of the interactions between the tumor and its stroma may shed light in the process of malignant tumor progression. The connection between mechanical and biochemical signals and how they play together with genetic and epigenetic alterations in tumor progression and metastasis formation is still elusive. Nonetheless, its knowledge may contribute substantially to the understanding of tumor progression and may change the current view dramatically as it has been reported for the role of the endothelium in tumor metastasis.

### Biophysical role of the endothelium in cancer cell invasion

The role of endothelial cells in tumor growth has long been presented as a clear picture showing that endothelial cells are necessary for a tumor to get larger than the critical size and to become definitively malignant (17). Several years ago, the “biochemical” role of endothelial cells in metastasis was well defined, as the endothelium has been described to act as a barrier for cancer cell invasion (Figure 4) (18-19). In 2008, the view of endothelial cells regarding their function in cancer metastasis has changed dramatically, as the endothelium has been reported to promote and increase the invasion of certain cancer cell lines into 3D-ECMs (Figure 4) (2). This important finding changed the view of the endothelium from a passive barrier that permits cancer cell invasion towards an endothelium that acts as an active modulator or enhancer of cancer cell invasion. However, there are still some open questions (Figure 5): What happened during the migration of
Conclusion

This article focused on the biomechanics of cancer cells, ECM tissue and adjacent endothelial cells and their impact on cancer cell invasion and metastasis formation. The ECM microenvironments provide a range of structural and molecular determinants that evoke substantial alterations in the migratory behavior of cancer cells. This may result in the modulation of cell adhesions, cytoskeletal reorganization and backward tailoring of the surrounding ECM that immediately modifies the shape of cancer cells, their contact guidance, the transmigration of the endothelium and invasion through connective tissue. The mechanical properties of the microenvironment and cytoskeletal remodeling dynamics operate through interactive pathways and control cellular behavior that is essential for the growth of tumors, the invasiveness of cancer cells, their transendothelial migratory behavior and finally their ability to metastasize. In particular, attention was drawn towards the importance of the endothelium to promote metastasis formation by enhancing cancer cell invasion. Taken together, the mechanical properties of the invasive cancer cells build the focus of a growing number of studies, whereas the role of the endothelial biomechanical properties in enhancing the invasiveness of cancer cells still needs further investigation. In summa-
Acknowledgement

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References


Meeting report of the 15. Joint Meeting "Signal Transduction – Receptors, Mediators and Genes" with the DGZ study group 'Signal Transduction'

Ralf Hass

Together with the Signal Transduction Society (STS), study groups of the German Society for Immunology (DGfI), the Society for Biochemistry and Molecular Biology (GBM) and the German Society for Cell Biology (DGZ) co-organized the 15th Joint Meeting "Signal Transduction – Receptors, Mediators and Genes" which was held at the Leonardo Hotel, Weimar, with a focus on 'Immune Cell Signaling'. As in recent years, this annual joint meeting was aimed to highlight a variety of state-of-the-art aspects in the fields of Biochemistry, Cell Biology, Pharmacology, Molecular Biology and Immunology. The meeting focus 'Immune Cell Signaling' was supported by members of the SYBILLA Initiative (Systems biology of T-cell activation in health and disease) from the European Union and from members of the Transregio SFB/TR52 (Transcriptional Programming of Individual T-cell subsets) from Würzburg and Mainz. The SYBILLA consortium represents a community of scientists from 11 nations focusing on T and B cell receptor activation and associated signal transduction cascades including regulation by post-translational modifications. Thus, a variety of keynote speakers from this consortium including E. Palmer (Basel), B. Alarcon (Madrid), O. Acuto (Oxford), W. Schamel (Freiburg) and R. Lahesmaa (Finnland) presented both, overview and original data on immune cell signaling. This topic was further supplemented by presentations from R. Hendriks (Rotterdam) about the control of B cell activation via B cell receptor signaling and by S. Ghosh (New York) about the role of NF-kB in T cell development and function.

Further workshops of the conference with corresponding keynote speakers included Tumor Biology (V. Poli, Torini, Italy), Pathogens and Disease (O. Fackler, Heidelberg), Growth Factors, Cytokines and Chemokines (M. Theilen, Bellinzona, Switzerland) as well as Cell Differentiation, Senescence and Cell Death (T. Schroeder, Munich). All workshops were introduced by the keynote speakers followed by a variety of short oral presentations which were selected by the appropriate workshop chair-persons from the submitted conference abstracts. Moreover, each session was concluded by a presentation from participating companies about new technologies and products in the field of signal transduction.

In addition to the workshops much attention was given to the presentation of meeting posters. Thus, each poster was introduced in a "One-minute-one-Transparency" plenary session followed by poster viewing and extensive discussions.

During a separate session the STS/CCS Honorary Medal 2011 has been awarded to the Director of the Salk Institute in San Diego, Prof. Anthony Rex (Tony) Hunter for his groundbreaking contributions to clarify fundamental mechanisms of signal transduction. Following the laudatio by Stephan Feller (Oxford, Editor-in-Chief of Cell Communication and Signaling) and the award ceremony by the members of the STS council (Frank Entschladen, Ottmar Janßen, Karlheinz Friedrich and Ralf Hass), Tony Hunter reported how he more or less accidentally discovered phosphotyrosine in 1979 and how this initial finding revolutionized the whole understanding of signal transduction cascades in physiological and pathological pathways within the last 30 years including the development of specific tyrosine kinase-associated therapeutics.

Like in every joint meeting within the past years, special attention was also given to young investigators and the STS together with the DGfI, GBM and DGZ study groups 'Signal Transduction' awarded 10 conference stipends of each 250,–€ and 5 poster prizes as well as a special GBM Innovation Award to Master students and PhD students. Moreover, the STS/CCS Science Award of 1,000. – €, which was sponsored by the company Biomal GmbH, was awarded to Ingo Schmitz, Braunschweig.

All together, it has been a very successful international conference again and detailed planning and preparations are already in progress for this year's 16th joint meeting 'Signal Transduction'. This forthcoming congress is scheduled for Nov. 5th to 7th, 2012 and the location will be again at the Leonardo Hotel in the history-associated town of Weimar. Details can be obtained from http://www.sigtrans.de. The special focus of this year’s meeting will be 'Tumor Biology'.

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Since several years, the study section “Cell Biology of viral infections” of the German Society of Virology (GfV) has been organizing an annual meeting held at the Ketschauer Hof in Deidesheim, Pfalz. This workshop aims to bring together cell biologists and virologists with the view to foster collaborations and cell biological analysis of virus infections. This year, we celebrated the 10th anniversary of the workshop, which was founded in 2001 by Beate Sodeik and Michael Kann at Zeilitzheim, Würzburg. To commemorate the anniversary, the traditional wine tasting was a particularly festive one, organized in the vineyards and the cellar of the winery “Reichsrat von Buhl”, Deidesheim.

The meeting took place September 21st to 23rd 2011 and focused on the “Modification of gene expression” (Fig. 1). Four keynote speakers were invited and presented overviews of their current work; they were a valuable contribution to a meeting that was up-to-date, very informative, well attended and lively. Interestingly, several keynote speakers did not know each other beforehand, which led to an unexpected side-effect, they made useful scientific contacts too. Meeting in a pleasant and rather intimate setting, the “cell-biologists” had ample time to converse and exchange ideas, which sparked their interest in mutual co-operations.

Stefan Hüttemaier is professor of Molecular Cell Biology and director of the Core Facility Imaging at the Martin-Luther-University Halle-Wittenberg. His lecture “A guided tour for mRNAs: Posttranscriptional control of cell migration” focused on the current knowledge and state-of-the-art analysis of the spatio-temporal distribution of mRNAs. Specific eukaryotic mRNAs seem to be inhibited translationally until they reach the subcellular region where their protein products are needed. A well known example is the transcript encoding β-actin (ACTB). β-actin is critically involved in modulation of plasmamembrane extensions like exploratory growth cones of neurons and lamellipodia of fibroblasts. To ensure the regulated expression of β-actin at sites highly active in cell migration, premature translation of the transported ACTB mRNA is prevented by the ACTB mRNA binding protein ZBP1 (Zipcode binding protein 1). Intracellular signalling activates regulatory kinases that phosphorylate ZBP1 which triggers its release from the ACTB mRNA and initiates regulated and local synthesis of β-actin. Additional mechanisms to control translation of mRNAs were described by Stefan Hüttemaier that enhance dynamics of the actin cytoskeleton and allow the control of cell polarization.

The research of Gunter Meister, head of the department of biochemistry at the University of Regensburg, focuses on the analysis of small regulatory or non-coding RNAs involved in regulation of gene expression. In his plenary talk on the “Mechanisms of microRNA-guided gene silencing” he provided an overview on various small RNAs, their biogenesis and influence on gene expression. Along with short interfering RNAs (siRNAs), microRNAs (miRNAs) are the most important members of this class of RNAs. While siRNAs are synthetic, exogenously introduced RNAs, miRNAs are processed products of cellular transripts. Gene repression by miRNAs is moderately efficient suggesting a role in fine-tuning the transcriptional activity to dynamically adjust for protein needs. The advent of deep sequencing led to the discovery of numerous other regulatory small RNAs originating from longer non-coding RNAs like tRNAs or snoRNAs, transposable elements or heterochromatic regions, a research area that is only evolving now. Future studies will focus on the mechanism of small regulatory RNAs to specifically regulate gene expression which makes them attractive tools for therapeutic interventions of diseases like cancer, neurodegeneration as well as viral infections.

Carsten Janke leads a research team at the
In his presentation Thomas Sternsdorf described the repertoire of SUMO conjugating enzymes accentuating the role of PML intra-nuclear bodies as potential sites where SUMO modification might take place under physiological conditions. He presented several new technical approaches that allow studying SUMO-modifications in cell based assays. These techniques included the use of in vitro semi-permeabilized or partially extracted cells (for in-tube assays) functionally preserving PML nuclear bodies and other cellular structures. The addition to such systems of bacterially expressed and purified substrates, of SUMO itself and an energy regenerating system was sufficient to restore SUMOylation indicating that the cellular structures provided the modifying machinery.

**Contributions by young scientists**

Aside from the invited plenary speakers, this workshop aimed to encourage the participation of young (and not so young) scientists from the field of virology by giving them the opportunity to present their work in the form of 20-minute oral presentations. Like last year, many participants came from outside of Germany underlining the quality of the research presented at and the dynamic nature of this workshop. These talks spanned a wide range of viral systems including alpha- beta and gamma-herpesviruses (HSV, HCMV, EBV), other DNA viruses (adenovirus, papilloma virus) as well as hepatitis virus B and C, influenza virus and some more exotic candidates. The presented topics covered all aspects from virus structure, entry mechanisms, replication, transcriptional control of viral and cellular genes during infections to virus assembly and egress. This wide range of topics stimulated considerable discussion involving several refreshing non-expert contributions of our invited plenary speakers, which gave rise to a new view of these topics, thus perfectly matching the scope of the workshop. These discussions did not stop in the sessions, but continued during, and particularly following, the wine tasting that ended in a social evening accompanied by the excellent food of the Ketschauer Hof.

It was clear from the talks that several state-of-the-art technologies have found their place in virus research that is focused around cell biological questions. In several sessions cutting-edge imaging data were presented, such as tomography and live cell imaging. For example, the use of single or dually tagged viruses, genetically modified with GFP and/or mCherry, and approaches using direct labeling with Alexa dyes of non-enveloped viruses was presented. The topics using imaging ranged from attachment, fusion and intracellular transport to virus induced membrane damage, morphogenesis and egress. This was an impressive demonstration how efficiently viruses can be used to highlight processes in living cells or at very high resolution. Others showed the use of cell based assays to reconstitute intracellular processes like virus docking and virus genome release using purified components in semi-permeabilized cells. This was followed by several talks on mechanisms related to viral and cellular gene expression, emphasizing the role of transcriptional and post-transcriptional control of viral and cellular genes as well as immune evasion strategies involving the modification of gene expression. Another group of talks encompassed more global approaches in form of large screening assays or transcription profiling approaches of viral or cellular miRNAs to identify novel restriction mechanisms of virus infections, but also to screen for nucleo-cytoplasmic transport signals. A lot of emphasis was also put on the use of primary cells showing a trend to use physiologically more relevant systems in viral research.

Lastly, as a new idea for this year's workshop, we selected three young scientists who gave excellent presentations, Sabrina Schreiner (“Control of adenoviral gene expression”, Heinrich-Pette-Institut, Hamburg), Diana Lieber (“Host miRNA profiling in alpha-herpesvirus replication”, Max von Pettenkofer Institute, Munich) and Martin Strehle (“Expression
Workshop 2012: “Nuclear structures and chromatin dynamics”

This year’s workshop will take place September 19th to 21st 2012 at the Ketschauer Hof in Deidesheim and is entitled “Nuclear structures and chromatin dynamics”. Generally, virally encoded proteins and RNAs often dramatically reprogram host gene expression while taking advantage of the host machinery for their own gene expression. Viruses that start their morphogenesis in the host nucleus annex this space for their propagation thereby restructuring the host chromatin. Despite these dramatic consequences for the host, surprisingly little is known how viruses achieve them. Recently, nuclear dynamics in viral infection, a field neglected for a long time, has attracted a lot of attention. To further boost research on this growing field and gain access to the state-of-the-art methodology, we invited three cell biologists specialized on various aspects of this year’s topic to give keynote lectures:

Gernot Längst is professor at the University of Regensburg. His research analyses how genomic DNA is highly compacted and organized into chromatin, while at the same time access of proteins involved in various DNA associated nuclear activities is guaranteed. Using biochemical methods, live cell imaging and deep sequencing the Längst lab analyses how various proteins and non-coding RNAs dynamically regulate chromatin compaction. With his keynote lecture “From local to global chromatin structures - Regulation by chromatin remodeling enzymes and non-coding RNA” Gernot Längst will provide insight into this fascinating research area.

The research of Cristina Cardoso, professor at the TU Darmstadt, aims at elucidating principles that enable and govern the dynamic organization of the cell nucleus. To analyse how the genetic and epigenetic information of the genome is replicated in a faithful and coordinated manner her laboratory applies high-resolution microscopy using fluorescent fusion proteins as well as other biochemical and biophysical approaches. Her talk on “DNA replication and repair, a 4D matter” will summarize work of her laboratory that has consequences for genome stability and cancer development, as well as cell differentiation.

Peter Hemmerich, professor at the FLI-Leibniz Jena, analyses structure and function of the cell nucleus with a particular focus on age-related alterations. He aims to determine dynamics and interactions as well as biophysical properties of proteins in their natural environment. To this end he applies high resolution and live microscopy in combination with several other techniques like photobleaching analysis of MHV68 miRNAs”, Helmholtz Centre, Munich). Among these three candidates, Diana Lieber was chosen (based on a random selection between the three candidates) to represent the workshop in an invited talk at the annual meeting of the GIV held in Essen earlier this year.
and -activation, and fluorescence correlation spectroscopy. In his talk entitled “Assessing protein dynamics in the nucleus: new clues on genome function” he will provide insight into the recent development in the field of nuclear dynamics and architecture.

Most importantly, we are very happy to welcome Prof. Harald zur Hausen who was awarded the nobel prize for physiology or medicine in 2008 for his life-long research on papillomavirus and their potential to induce cancer. We look forward to host Prof. zur Hausen who will present a lecture of honor.

We would now like to invite researchers of the Cell biology and Virology fields at all levels of their career to join us in Deidesheim and to provide fascinating contributions and to engage in lively discussions.

Details for the workshop can be obtained at the website of the GfV or by directly contacting the organizers.

The DGZ welcomes the following new members:

Eleni CHRISTODOULOU
Dr. Dorothee DORMANN
Melanie GRÄSSL
Prof. Dr. Josef KÄS
Judith KOLIWER
Manuela KOWALCZYK

Dr. Oliver ROCKS
Nina SCHULZE
Prof. Rüdiger SIMON
Prof. Dr. Theresia STRADAL
Nico ULLRICH, M.sc.
Monika ZUBEROVA

Missing members:

We have no valid address from the members listed below. If anybody can help us in this respect, please send a message to the DGZ office at dgz@dkfz.de.

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Koku Zikpi Adjogble
Marwan Al Falah
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Horst Waldvogel
Membership Application
I hereby declare my membership with immediate effect to the German Society for Cell Biology. The DGZ annual fee of EUR 52.00 for full members, EUR 35.00 for double members (combined membership in DGZ & GBM – Society for Biochemistry and Molecular Biology) and EUR 18.00 for student members is collected at the beginning of the year.

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anti-TIP 47/ PP17, C & N-terminus specific
anti-LDL-Receptor
anti-p62, C- & N-terminus specific
anti-p97 ATPase
anti-26S Proteasome
anti-p53

Antibodies to Cell Adhesion Proteins
anti-p0071 Protein, mouse monoclonal
anti-p0071 Protein, guinea pig serum
anti-ARVCF, mouse monoclonal
anti-HEA125 (Ep-CAM), mouse monoclonal
anti-Desmocollin 1-3, mouse monoclonals
anti-Desmoglein 1-3, mouse monoclonals
anti-Desmoglein 4, guinea pig serum
anti-Desmoplin, mouse monoclonal
anti-MyoZap, mouse monoclonal
anti-Plakophillin 1-3, mouse monoclonals

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