Cell News

Newsletter of the German Society for Cell Biology

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Physics of Cancer

Join us in Leipzig, September 24 - 27 2013

DGZ

CONTENT

Newsletter of the German Society for Cell Biology

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Cover image: Müller glial cells act as living optical fibers, transporting light through the inverted retina of vertebrates. Wilh their funnel-shaped endfeet, Müller cells collect light at the retinal surface and guide it to photoreceptor cells on the opposite side. Images are thus transmitted through optically distorting tissue (K. Franze et al. (2007) *Proc. Natl. Acad. Sci. USA 104, 8287–8292*). Image courtesy of Jens Grosche.

Consolidation of financial affairs

Critical questions have been raised at the last DGZ members meeting in Heidelberg in March concerning the financial situation of the society and the support of young scientists. The society was facing a variety of structural changes within the last five years including the organization and co-funding of special interest meetings, a new web appearance, and a complete new format and label of our members' Journal Cell News. These measures very much improved the national and international visibility of the German Society for Cell Biology and highly supported the cell biology community in Germany. However, the measures were cost intensive and over the last years continuously consumed most of the savings of the DGZ. The new executive board, which took over a year ago was challenged from the beginning to keep up with the new ideas and to consolidate finances at the same time. We are working since then to navigate the society in a way that we can keep the German Society for Cell Biology a modern, attractive, and international interacting society. We will therefore go on to support two special interest meetings per year, one in spring and one in fall. We hope to establish this routine in 2014. The special interest meetings should have strong poster sessions and include as many poster short talks as possible to support young scientists. As soon as the financial situation has improved (we foresee a positive financial development this year), we plan to revive/promote our travel grant system for graduate students and younger post-docs for DGZ meetings and selected international meetings such as the ASCB meeting.

Cell News goes online

We also will go on with our members' journal in a modern, electronic format. Also given the fact that the actual costs of the Journal used up one third of the member fees, we decided to develop an online journal with all the advances of novel information technologies. Cell News has developed over the years into a valuable resource of information as well as excellent platform for upcoming and established Cell Biologists to present themselves and their ongoing research. We feel that the new online format will allow fully exploiting all the advantages of modern information technologies. For instance, we will be able to show videos and supplements, and the new format will be increasingly attractive for advertising companies. The novel Cell News format will have its own homepage, PDF files of all articles, and will include a new Cell News App for smart phones and tablet computers.

Despite our efforts to re-structure our annual conference schedules and to modernize the publishing mode of Cell News, it was also necessary to slightly increase our member fees (NEW: EUR 60 full member, EUR 40 double member, EUR 20 student member), which was fully supported by participants of our last members meeting in March. Together, we are convinced that all these activities will consolidate the society's financial situation and thus ensure our various future activities.

Eugen Kerkhoff

Eliminating the impact of the impact factor: The San Francisco Declaration on Research Assessment

When you navigate through the homepage of the *Journal of Cell Biology* you will learn that chief editor Tom Misteli's comment on the call against the *'numerical measure of a scientists work'* using the Impact Factor (IF) climbed up to the currently most read contribution in JCB. EMBOs chief Editor Bernd Pulverer caught up and wrote an equally excellent statement in the last issue of *EMBO Journal*.

Both editorial comments explain that during last year's American Society of Cell Biology meeting in San Francisco *'a group of prominent journal editors and publishers of scholarly journals, as well as representatives from major funding agencies and research institutions'* spoke up *'as one voice to highlight the limitations of the IF and to call for a concerted effort to improve the ways scientific output is assessed by funding agencies, academic institutions, and scientists themselves.'*
Their consultations yielded *The San Francisco Declaration on Research Assessment*, found at the URL am.ascb.org/dora/ of the ASCB. The declaration raises a number of issues against the abuse of the IF, point by point, suggested to be considered by funding agencies, institutions, publishers, organizations that supply metrics, and ourselves, the researchers. So far, the declaration has been signed by close to 8000 individual scientists and 300 organizations, among them the German Society of Cell Biology and the VBIO. Board and Scientific Advisors of the DGZ unanimously decided to do so and we hope that many of you as DGZ members or just scientists interested in cell biology will follow our example.

DGZ Board Members

Mitgliederversammlung der DGZ am 21. März 2013 in Heidelberg

Teilnehmer:
Vorstandsmitglieder Eugen Kerkhoff, Ralph Gräf, Oliver Gruss und Klemens Rottner, sowie Harald Herrmann-Lerdon und weitere Beiratsmitglieder, sowie DGZ-Mitglieder laut Anwesenheitsliste.

TOP 1: Begrüßung durch Eugen Kerkhoff und kurze Skizzierung derzeitiger und zukünftiger Aktivitäten

TOP 2: Finanzen

Oliver Gruss beschreibt in seiner Funktion als Geschäftsführer ausführlich die derzeitige Finanzsituation sowie die Entwicklung in jüngsten Jahren und die Aussichten für das kommende Jahr. Hauptthema dabei waren die gestiegenen Haushaltsbelastungen in den Jahren 2011 und vor allem 2012 aufgrund einer hohen Anzahl an unter dem Dach der DGZ ausgerichteten Tagungen. Daraus entstand ein Defizit, das jedoch durch DGZ-Rücklagen ausgeglichen werden konnte. Da das Finanzpolster dadurch geschrumpft ist, sollte in den kommenden Jahren die Finanzsituation restriktiver im Auge behalten werden.
Es wurde den Mitgliedern daher ein von Vorstand und Beirat in ihren Sitzungen vorbereiteter Katalog von Maßnahmen erörtert, die zu einer deutlichen Entspannung der Finanzsituation führen sollten. Beispielsweise wird es im Jahre 2013 deutlich weniger Tagungen geben.
Weiterhin wurden die Mitglieder dazu befragt, ob ab 2014 der Jahresbeitrag von derzeit EUR 52 auf EUR 60 oder auf EUR 65 angehoben werden soll. Eine erste Abstimmung ergab eine Mehrheit für EUR 60, und dies war auch nach darauffolgender, lebhafter Diskussion bei einer zweiten Abstimmung der Fall.
Im Zuge dessen beschloss der Vorstand, den Mitgliedsbeitrag ab 2014 auf EUR 60 für Vollmitglieder zu erhöhen! Der Beitrag für DGZ/GBM-Doppelmitglieder wird auf EUR 40 und der für Studentische Mitglieder nur moderat von EUR 18 auf EUR 20 angehoben.

EINNAHMEN €		AUSGABEN €	
Mitgliedsbeiträge	46.990,50	Bankkosten	1.063,37
Spenden, Preisgelder	16.000,00	Retoure Mitgliedsbeiträge	913,00
Zinsen	1.418,73	Reisekosten	5.977,72
DGZ-Mitgliederzeitschrift	19.361,30	DGZ-Mitgliederzeitschrift	37.507,90
Firmen-Links (Homepage)	2.439,51	Spenden, Preisgelder, Reisestipendien	26.330,95
DGZ-Tagungen	45.650,04	DGZ-Tagungen	85.191,76
Überträge	7.204,00	Bürokosten (u.a. Gehalt Sekretärin, DGZ-Homepage)	692,21
Sonstige	2.500,00	Überträge	7.204,00
		Sonstige	12.526,81
Summe der Einnahmen:	**141.564,08**	**Summe der Ausgaben:**	**177.407,72**
Guthaben am 31.12.2011:	147.218,71	Guthaben am 31.12.2012:	111.375,07
Guthaben DGZ:	84.378,66	Guthaben DGZ:	52.846,51
Werner Risau Preis:	62.840,05	Werner Risau Preis:	58.528,56

Die Einnahmen und Ausgaben wurden am 21.02.2013 von den beiden Kassenprüfern Prof. Dr. Marie-Christine Dabauvalle und Prof. Dr. Hans-Georg Mannherz in Heidelberg geprüft und für richtig befunden.

TOP 3: Entlastung des Vorstands

Auf Antrag von Harald Herrmann-Lerdon wurde der Vorstand entlastet (eine Enthaltung).

Forces and flows: Actomyosin ring function in zebrafish epiboly

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Introduction

The development of an embryo from a single cell into a complex organism is accompanied by a rich variety of morphogenetic events. Tissues have to spread, fold or contract in a precisely patterned scheme to ensure the functioning form of the embryo. On a molecular scale filamentous actin and non-muscle myosin-2 have been characterized early on as key players involved in cell and tissue morphogenesis *(1, 2)*. Together with a myriad of other components they form a dense meshwork that comprises the actomyosin cortex *(3-5)*. While this network promotes shape changes on the cellular and tissue scale, the mechanisms of force generation through actomyosin networks remain unresolved in most instances. Only recently have advances in imaging and force probing techniques allowed to gain a better mechanistic insight into how molecular forces integrate into cell and tissue deformation and flow *(6-8)*. For example, during *Drosophila* germband elongation orientation-specific enrichment of myosin-2 at cellular boundaries of the epithelium drives junctional remodeling in a local cell intercalation process, which integrates on the tissue scale to an effective elongation of the epithelium *(9)*. In zebrafish gastrulation down-regulation of the actomyosin cortex at cellular contacts control contact expansion and promote in an interplay with differential adhesion cell sorting for the establishment of distinct germ layers *(10)*.

How myosin contractile activity generates flow is best understood in the *C. elegans* zygote. Newton's laws must be obeyed at all scales, and flows are associated with mechanical tension in the cortex. Importantly, force generation at the molecular scale through the activity of non-muscle myosin-2 can give rise to isotropic active tension (also referred to as contractility). An imbalance in myosin concentration generates a gradient in active tension in the direction in which myosin concentration is changing. The active tension gradient in turn drives actomyosin cortical flow in a manner akin to how pressure gradients drive Stokes' flow in a highly viscous fluid *(11, 12)*. A fluid description is appropriate because cortical constituents turn over multiple times during flow, rendering the material effectively viscous. It is important to identify and measure mesoscale physical properties of the cortex, since they determine the behavior of the cortex at larger scales. For example, cortical viscosity determines over what distance myosin activity can drive flow *(11)*.

While some aspects of actomyosin mechanics at larger scales have been identified, much remains to be explained. For example, apical constriction in the presumptive mesoderm of *Drosophila* embryos does not occur as previously thought through a continuous contraction processes of cortical junctions, but is mediated through contractive pulses of the apical actomyosin network *(13)*. What governs the period of these pulsatile actomyosin flows and how the flows translate into effective force generation on the tissue scale, remain exciting open questions. For example, in *C. elegans* gastrulation, a molecular clutch mechanism has been explored in which actomyosin flows at the apical cortex of endodermal precursor cells precede the actual shrinkage of the apical surface. Only upon mechanically coupling the centripedal cortical flows through a cadherin-catenin complex to the cellular junctions, actomyosin contraction is efficiently translated into cellular shape changes, eventually triggering endodermal internalization through apical constriction *(14)*. A full understanding of actomyosin dynamics and mechanics can only be provided when all relevant mesoscale physical properties have been identified by theory and measured in experiments, and their relation with deformation and flow have been characterized by theory and tested in experiments. This will be a challenging task given the wealth of different morphological processes. Nevertheless, it will be essential to find unifying themes to bring us a step closer to understand how biological form is controlled during embryonic development.

In this article we will focus on the first major morphogenetic movement in the life of a zebrafish embryo, the epiboly movement. This is a particular exciting example of tissue morphogenesis as it involves the concerted spreading of an epithelial tissue and an adjacent actomyosin structure of embryonic dimension, which is situated within a continuous syncytium. To some extent, epi-

thelial spreading during zebrafish epiboly thus directly links force generation of a single, albeit large, cellular structure with morphogenetic shape changes of an entire epithelial tissue. We will outline how a combination of experiments together with a theoretical description of actomyosin network mechanics, has provided insight into the biophysical mechanisms driving it (15).

Zebrafish epiboly

Zebrafish gastrulation is an excellent assay system for studying the biophysical mechanisms of global tissue organization. Major morphogenetic movements result in the formation of distinct germ layers (ectoderm, mesoderm, endoderm) and the establishment of an embryonic body axis *(16)* during the course of gastrulation taking place between 4 hours and 10 hours post fertilization (hpf). Epiboly comprises a central gastrulation movements, referring to the thinning of the blastoderm as it engulfs the underlying yolk cell in an animal-to-vegetal (AV) directed spreading (Fig. 1). Three different types of tissues are involved in this process. The deep cells, which will form the embryo proper and are initially residing at the animal pole on top of the yolk cell, and two extra-embryonic tissues - the yolk syncytial layer (YSL), a thin multinucleated cytoplasmic layer at the surface of the yolk sac, and the enveloping cell layer (EVL), a simple squamous epithelial cell layer that covers the deep cells and is connected at its margin with the YSL *(17)*. The molecular and cellular processes by which these different cell types/tissues undergo epiboly movements have only begun to be understood *(18)*.

The initiation phase of epiboly, also referred to as 'doming' (4.33 hpf), is characterized by a drastic reorganization of the blastomere mass, as the deep cells radial intercalate to flatten the tissue. Simultaneous with the deep cell rearrangements, the yolk cell underneath bulges towards the animal pole in a doming movement *(19)*. Cell motility of the deep cells, recently shown to depend on E-Cadherin endosomal trafficking *(20)*, is critical in this process. Whether radial intercalation during doming is driven by an active remodeling of the deep cells *(21)* or is rather a passive consequence of pushing forces from the bulging yolk cell *(22)* remains unknown. Importantly, for subsequent phases of epiboly, EVL movements are independent from deep cell epiboly as E-Cadherin loss of function leads to an arrest of deep cell epiboly at the equator of the embryo, while the EVL progresses normally *(23)*; more than doubling its surface area over the course of a few hours. What is the driving mechanism for this massive epithelial spreading?

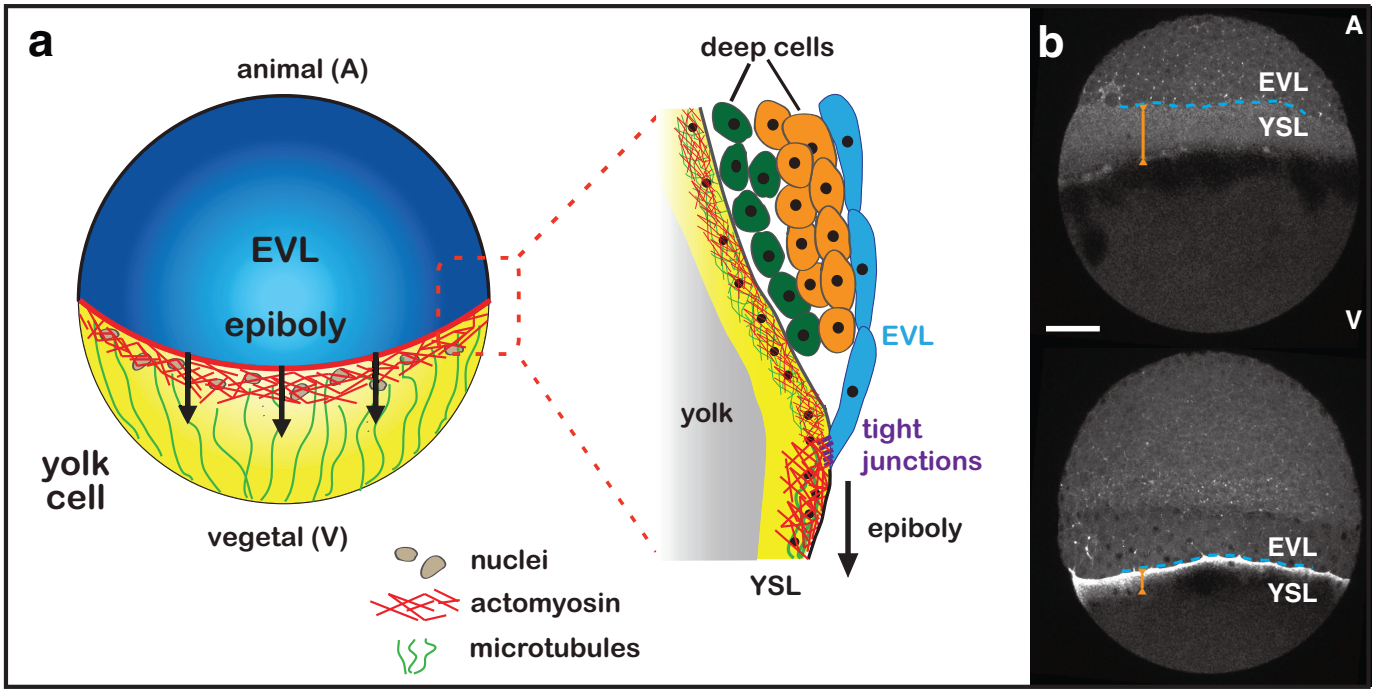

Figure 1: Actomyosin ring formation during zebrafish epiboly.
(a) (Left) A schematic depiction of a zebrafish embryo at 50% epiboly. Epiboly movements refer to the animal-to-vegetal (AV) spreading of the tissues over the yolk cell. (Right) Illustrative cross section of the tissues involved in epiboly. Deep cells give rise to the embryo proper and are enveloped by the EVL. The EVL is connected at its margin to the YSL, a thin cytoplasmic layer containing nuclei and cytoskeletal structures.
(b) Myosin-2 localization in Tg(actb2:myl12.1-eGFP) embryos: At the onset of epiboly, a broad diffuse actomyosin band (orange bar) forms within the YSL (40% epiboly, upper panel). As epiboly proceeds the band constricts to from a disctinct ring-like structure (70 % epiboly, lower panel). Scale bar, 50 µm. Adapted from (15).

During early phases of epiboly (30-50% epiboly, 4.66-5.25 hpf), marginal EVL cells exhibit actin-rich filopodia protrusion and have been therefore proposed to participate actively in the spreading *(24)*. In contrast, filopodia are no longer detected once the EVL margin reaches the equator of the sphere (50% epiboly) *(18, 25)*. At the same time (30-40% epiboly) a ring-like actomyosin structure forms within the YSL adjacent to the EVL margin. It spans around the circumference of the yolk cell and becomes increasingly pronounced *(17, 25)*. The marginal EVL cells are connected to the YSL via tight junctions *(17, 26)*. Altering myosin activity specifically within the YSL via the mitogen-activated protein (MAPK) pathway components traf2/nika and mapkapk2 suggest a critical role of the YSL actomyosin ring for EVL epiboly *(17, 27)*. To test if the YSL actomyosin ring generates the forces that drive EVL movements, we utilized a UV-Laser ablation setup to locally disrupt the actomyosin ring in close proximity of the EVL cells *(15)*. We found considerable delays in EVL cell movements adjacent to the ablation site, supporting the role of the actomyosin ring as a driving force for the epithelial spreading. However, the key question still remains: How does the actomyosin ring generate the necessary force to accomplish this task?

Actomyosin ring contraction

Contractile actomyosin rings function in many different morphogenetic processes. On the cellular scale, actomyosin rings are assembled at the equatorial plane of dividing cells to drive cytokinesis *(28)*. They also participate in the apical constriction of invaginating cells *(29)*. Supracellular actomyosin rings form upon wounding of an epithelium, and they promote the closing of the wound *(30)*. This is also how 'dorsal closure' proceeds in *Drosophila*. Here, a supracellular actomyosin cable contributes to the closing of an epithelium on the dorsal side of the embryo *(31, 32)*. The common view of how actomyosin rings function in these processes is through a purse-string mechanism. Contractile myosin motor protein activity on the circular actin structure causes the ring to radially constrict. This triggers shape changes upon the cell/tissue structures to which it is associated. One possible mechanism for the function of the actomyosin ring in zebrafish epiboly therefore is that myosin motor function generates active tension along the circumference of the ring (circumferential active tension), which, due to the spherical geometry of the embryo gives rise to a force pulling on the EVL margin once the ring has passed the equator.

This hypothesis concerns cell and tissue mechanics underlying epiboly. Testing it requires in vivo mechanical measurements of the tension within the epibolyzing actomyosin ring. Here, cortical laser ablation (COLA) has proven to be a very versatile tool *(9, 11, 14, 32)*. The basic principle of cortical laser ablation is that a cortex under tension will rapidly recoil in response to a cut, with a recoil velocity that is proportional to the mechanical tension present before the cut. As such, COLA allows to compare relative tension along different orientations or at different times. We systematically mapped the tension within the actomyosin ring over the course of zebrafish epiboly by use of a pulsed UV laser. In agreement with the hypothesis that the YSL actomyosin ring acts as a purse-string, we measured significant circumferential tension within the ring (Fig. 2a), which depends on myosin-2 activity and increases during epiboly progression *(15)*. Due to the spherical geometry of the yolk cell, circumferential active tension results in a net force pulling on the EVL margin, which is zero when the ring is at the equator and grows as the ring approaches the vegetal pole. Consequently, when the actomyosin ring is near the equator of the yolk cell, tension within the ring in the animal-vegetal (AV) direction is expected to be much smaller than circumferential tension *(15)*.

To our surprise we found that the AV tension within the ring at 60-70% epiboly (Fig. 2a) is roughly half of the circumferential tension, and remains constant throughout epiboly (15). These results are incompatible with the actomyosin ring functioning as a simple purse-string only. To find out where the additional tension in the AV direction comes from, we turned towards analyzing actin and myosin dynamics. Interestingly, we observed that the formation of the ring is accompanied by substantial flow of actin and myosin from vegetal parts of the yolk cell towards the margin of the EVL, and in a direction opposite to those of epiboly movements (Fig. 2b). These retrograde cortical flows are initially slow and long-ranged, but increase their speed at later stages of epiboly *(15)*.

Cortical flows are a wide spread phenomena in animal cell morphogenesis *(3)*. The emergence of cortical flows has been associated with gradients of myosin contractility and recent studies in different model organisms have highlighted their pivotal role in fundamental morphogenetic processes ranging from cellular polarization to cell intercalation or tissue invagination *(11, 13, 14, 33, 34)*. To elucidate how retrograde cortical flows might contribute to zebrafish epiboly, we require a biophysical framework that identifies the relevant mesoscale physical properties and their relation with deformation and flow. To this end we developed a theoretical description of actomyosin network mechanics in the YSL as well as in the EVL tissue. This description is conceptually similar to the one utilized for unraveling the physical basis of cortical flow in the *C. elegans* zygote *(11)*.

Flow-friction, a crawling motor

The YSL cortex is a thin (1-2 µm) network on the surface of the yolk cell that expands along the AV direction with a width of 30-80 µm and spans around the circumference of the spherical embryo having a radius of roughly 350 µm. It consists of a highly dynamic network of cross-linked actin filaments, upon which myosin motor proteins can exert active forces through the consumption of ATP *(8)*. The dynamics of this collective network are

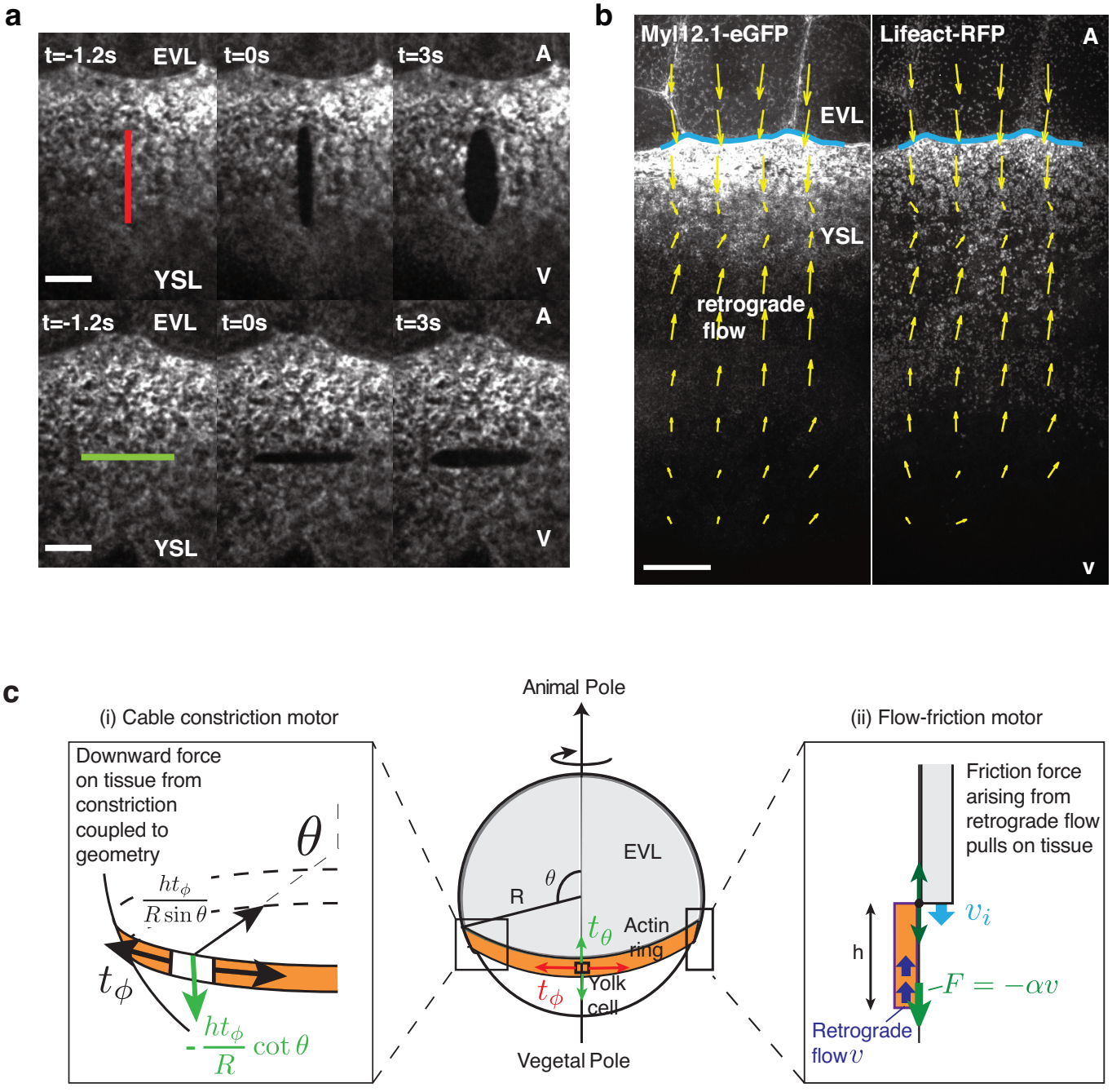

Figure 2: Cortical tension and actomyosin flow within the YSL suggest two modes of actomyosin ring propulsion.
(a) Laser ablation experiments in the YSL actomyosin band of Tg(actb2:myl12.1-eGFP) embryos at 65% epiboly reveal substantial circumferential tension (red, upper panel) as well as tension along the AV axis (green, lower panel). Scale bars, 10 µm.
(b) In Tg(actb2:myl12.1-eGFP) embryos injected with lifeact-RFP mRNA actomyosin band progression at 65% epiboly is accompanied by retrograde flow of myosin (left) and actin (right) from vegetal parts of the yolk cell into the EVL margin (blue line). Particle image velocimetry (PIV) allows for quantification of the flow velocity field (yellow arrows). Scale bar, 20 µm.
(c) Theory of epiboly movements. Modeling the EVL and YSL tissues as thin active viscous layers reveal a two-fold contribution of the actomyosin ring to EVL epiboly. (i) Active tension along the circumference of the actomyosin ring couples to the spherical geometry of the embryo resulting in a net force towards the closest pole. (ii) Retrograde actomyosin flows are resisted by friction against a substrate and exert a pulling force onto the EVL tissue. Adapted from (15).

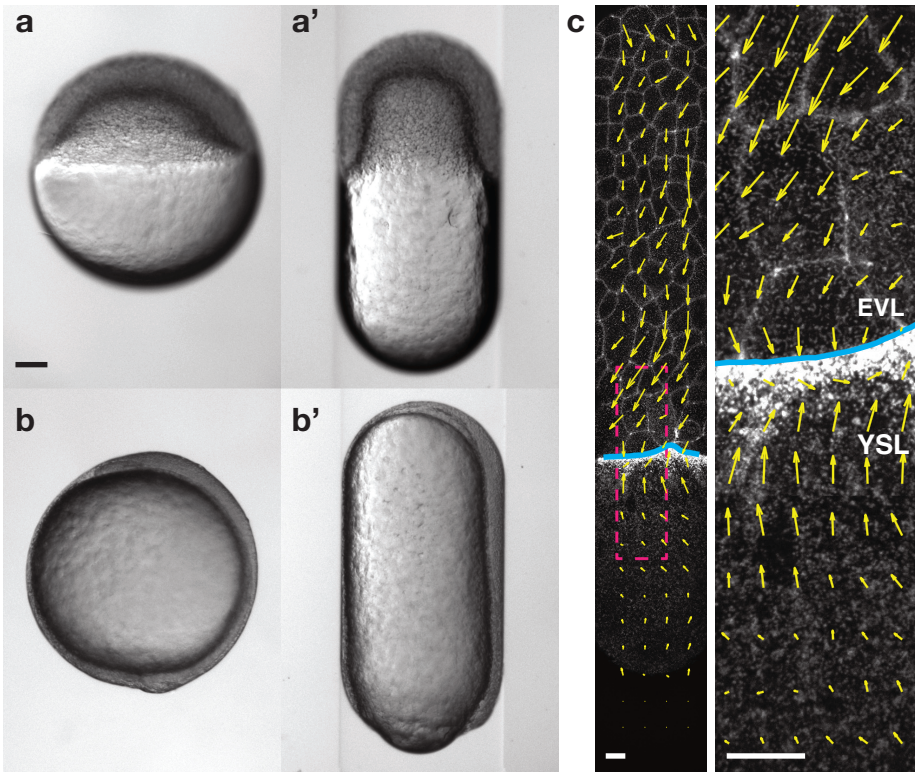

Figure 2: Cable-constriction of the actomyosin band is not necessary for EVL epiboly.
a (a-b) Aspiration of Tg(actb2:myl12.1-eGFP) embryos into agarose tube deform embryos towards a cylindrical shape. Brightfield images were taken of control embryos and cylindrical embryos at 30% epiboly (a,a') and at 100% epiboly (b,b'). c) Actomyosin band formation, cortical flows and epiboly progression are largely unaffected in cylindrical embryos at 60-70% epiboly. Cortical flow velocities are quantified using PIV (yellow arrows). Scale bars, 25 µm. Adapted from (15).

characterized by cortical flows and circumferential constriction; both occurring with velocities on the order of µm/min. On macroscopic scales relevant for epiboly progression it is sufficient to abstract from the molecular details and describe the cortex on a coarse-grained or mesoscale level. Here, macroscopic parameters such as active tension, viscosity or friction effectively capture the large-scale contributions of processes of molecular (e.g. myosin motor activity) or cellular (e.g. cell division/rearrangements) origin *(12, 35, 36)*. Our theoretical model identifies two modes of YSL actomyosin ring propulsion in epiboly (Fig. 2c). First, circumferential tension within the actomyosin ring couples to the geometry of the yolk sphere and results in a net pulling force onto the EVL towards the vegetal pole. This 'cable-constriction' mechanism can drive epiboly once the ring has passed the equator and accounts in the absence of friction for the total force of the ring exerted upon the EVL. In addition, if retrograde cortical flows within the YSL are resisted by friction with the yolk plasma membrane or cytoplasm, these flows will give rise to a geometry-independent mechanism, where the ring self-propels in a crawling fashion (also referred to as 'flow-friction motor'). Importantly, friction-resisted cortical flows generate additional AV tension in the YSL actomyosin ring in consistency with the low degree of tension anisotropy measured in the network. Moreover, our theoretical description accurately predicts experimentally measured flow profiles within the EVL and the actomyosin ring, as well as the relative tension obtain from laser ablation, only when taking significant friction into account *(15)*.

To test whether the newly predicted 'flow-friction' mode of propulsion would be sufficient to drive epiboly movements, we sought to change the geometry of the embryo such that the ring always faces the situation it encounters at the equator: At this location, cable constriction will not lead to a net pulling force on the connected EVL tissue. We achieved this by squeezing embryos into a cylindrical shape, through aspiration into small agarose tubes (d = 500 µm, Fig. 3a,b). Due to the lack of curvature along the AV direction in this condition, a 'cable-constriction' mechanism now cannot exert a pulling force onto the EVL. Surprisingly, epiboly movements are largely unaffected and proceed with velocities similar to spherical control embryos (Fig. 3c, *(15)*). This shows that cable-constriction is not essential for YSL actomyosin ring propulsion and indicates that the crawling mechanism achieved by actomyosin flow is sufficient to drive EVL epiboly during zebrafish gastrulation.

Conclusions

The insight we gained from studying the force generating mechanism for zebrafish epiboly have important implications for the general function of actomyosin rings in morphogenesis. Whereas actomyosin ring are commonly assumed to function through circumferential contraction, we found that in the case of zebrafish epiboly friction-resisted actomyosin flows can represent an equally important force generating process. Future studies analyzing the mechanics and dynamics of actomyosin rings in other processes such as cell division, wound healing or apical constriction will be needed to unravel potential contributions of circumferential contraction versus friction-resisted flows into the ring.

Moreover, it would be interesting to explore to what extent actomyosin rings might have evolved to optimize the use of a

'cable-constriction' versus a 'flow-friction' motor in the context of their biological function. In the case of epiboly, a geometry-independent crawling type of 'flow-friction' is advantageous, because it contributes to the epiboly movement even before the ring has reached the equator. On the contrary for cytokinesis, while friction-resisted flows along the width of the ring could play a role in the positioning of the ring or its assembly (37-39) cable-constriction along the circumference is needed for its main task to physically divide the cell.

To conclude, the study discussed here highlights the importance of identifying and understanding the biophysical material laws by which actomyosin-based flow and deformation of cells and tissues arise. These laws will provide us with the relevant coarse-grained and mesoscale biophysical parameters which determine large-scale dynamics, and only they will allow us to understand how particular morphogenetic processes arise and proceed. Therefore, an essential task for the future is to understand how the relevant physical properties emerge from molecular activities within the actomyosin cortex.

Acknowledgements

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For CV see Stephan Grill's article in Cell News 4/2011 (p. 33)

Notch-dependent VEGFR3 upregulation enables angiogenesis without VEGF–VEGFR2 signalling

Rui Benedito

Blood and lymphatic vessels have been the subject of intense investigation because they are seen as important therapeutic targets in many types of cancer and in cardiovascular diseases[1,2]. The formation of new blood vessels from pre-existing vessels, a process named angiogenesis, requires the coordination of several cellular and signalling mechanisms. The inner lining of blood vessels is formed by endothelial cells that express several surface receptors that are able to sense different extracellular molecular cues. The vascular endothelial growth factor (VEGF) family of secreted ligands and receptors are among the most important and specific regulators of endothelial cell sprouting, proliferation and survival in a variety of organs and pathological processes[2,3]. In addition to being influenced by external factors, endothelial cells also have endogenous signalling mechanisms that can modulate their response to the surrounding environment. One such mechanism is the Notch signalling pathway, where both ligands and receptors are transmembrane proteins and upon cell-to-cell ligand-receptor activation, elicit a transcriptional programme that leads to a change in the cell status and behaviour of adjacent cells[4].

Mutant mice lacking VEGF or Notch function in endothelial cells die very early during development due to severe defects in the genesis of the first embryonic vessels[5-7]. VEGF is required for vascular sprouting and proliferation whereas activation of Notch by the ligand Dll4 inhibits these processes[4,8].

The efficient formation of new blood vessels requires properly shaped angiogenic gradients that can activate only a few specific endothelial cells within an existing vascular network. These first responsive cells, take the leading position in a vascular sprout and are known as endothelial tip cells. The other adjacent endothelial cells follow the leader cell and are named by endothelial stalk cells.

In the last years, several studies suggested that VEGF could induce expression of the Notch ligand Dll4 in tip cells and activation of Notch receptors by this ligand then repressed the transcription of the most important VEGF receptor (VEGFR2/Kdr) in adjacent stalk cells, reducing there angiogenic activity and ability to outcompete tip cells in the cellular struggle to the leading position. These results, led to the interpretation that this negative feedback mechanism, of VEGF-to-Dll4 activation in tip cells and Notch-to-VEGFR2 inhibition in stalk cells, is what allows the selection of endothelial cells for sprouting and the properly balanced heterogenous response of the endothelium to the angiogenic stimuli[3,8].

Most of the experiments supporting this strong retroregulation between VEGF and Notch were performed with defined endothelial cell lines (HUVECs) *in vitro*[9-11]. However when we quantified the amplitude of regulation *in vivo* in animals with induced deletion of *Vegfr2* in endothelial cells (*Vegfr2iDEC*), we observed only a small decrease in Dll4 expression (Fig. 1a, b). We also observed that when the Notch downstream transcriptional program is impaired in endothelial cells, by deleting the transcription factor *Rbpj* (*Rbpji*^{DEC}), there is no significant difference in *Vegfr2* transcription, protein levels or kinase activity when compared with other genes expressed in endothelial cells and not regulated by Notch, like Cdh5, or genes known to be regulated by Notch in endothelial cells like Dll4 or Hey1 (Fig. 1b, c).

These results showed that endothelial cells with no VEGF/VEGFR2 signalling still express the Notch ligand Dll4 and hence still have Notch signalling, therefore we decided to block Notch activity in vessels without VEGF signalling to see if that elicited any endothelial response. According to the previous model, where it was assumed that Notch controls the sensitivity of cells to VEGF, we would predict that endothelial cells without VEGF signalling cannot sprout or proliferate, independently of the existing Notch activity. However, we found that when we delete genes important for Notch signalling in endothelial cells (*Dll4* or *Rbpj*), these suddenly become much more resistant to the inhibition of VEGF or deletion of *Vegfr2* (Fig. 1d, e). Our results show that normally angiogenic endothelial cells are very sensitive to VEGF levels, but in the absence of Notch they can still proliferate and sprout even when VEGF signalling is impaired.

Apart from Vegfr2, previous studies showed that Notch can also negatively regulate the transcription of another receptor from

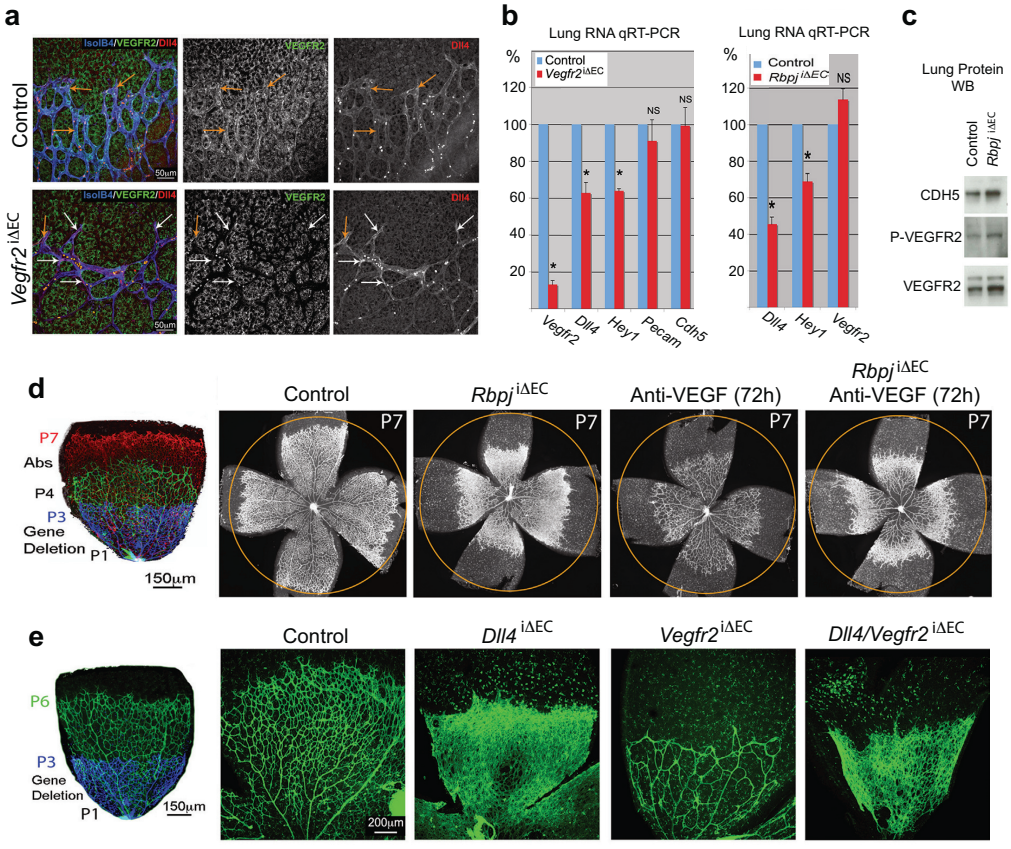

Figure 1
(a) Whole-mount triple immunofluorescence for Isolectin B4 (blue), Dll4 (red) and VEGFR2 (green) of P6 control and *Vegfr2*$^{i\Delta EC}$ retinas. Deletion of *Vegfr2* for 5 days strongly compromises angiogenesis but not Dll4 expression. Arrows indicate endothelial cells with (orange) and devoid of VEGFR2 (white).
(b) qRT-PCR analysis of *Vegfr2*$^{i\Delta EC}$ and *Rbpj*$^{i\Delta EC}$ P6 mouse lungs for the indicated transcripts showing that there is *Dll4* and *Hey1* expression in endothelial cells with *Vegfr2* deletion and that there is no significant difference in *Vegfr2* expression in endothelial cells after impairment of the Notch downstream transcriptional activity (*Rbpj*$^{i\Delta EC}$). Error bars represent s.e.m.; Asterisk, P < 0.001; NS, not statistically significant.
(c) Western blot analysis of *Rbpj*$^{i\Delta EC}$ P6 mouse lungs for the indicated proteins showing no significant difference in total or active VEGFR2 in these mutants. VE-cadherin (Cdh5) protein is not regulated by Notch and reflects the total endothelial content of both samples.
(d) Isolectin B4–stained control and *Rbpj*$^{i\Delta EC}$ P7 retinas (tamoxifen administration from P1 to P3) treated with control IgG or anti–VEGF-A antibodies from P4 to P7 (72 hours). Orange circles facilitate comparison of vascular progression.
(e) Induction of EC–specific *Dll4* or *Vegfr2* deletion from P1 to P3 and analysis at P6. Angiogenesis in *Dll4/Vegfr2*$^{i\Delta EC}$ and *Dll4*$^{i\Delta EC}$ mutants is strongly enhanced compared to control and *Vegfr2*$^{i\Delta EC}$ retinas suggesting that impairment of Notch signalling turns endothelial cells angiogenically active independently of VEGF/VEGFR2 signalling.

the VEGF family, Vegfr3/Flt4[12,13]. In contrast to VEGFR2, this receptor does not bind VEGF. It only binds VEGFC and VEGFD and these ligands are known to be very important for lymphatic vascular development but in most contexts they are not so important for blood vessel development[14], unlike the VEGF/VEGFR2 signalling axis that is a very powerful regulator of blood vessel development. For this reason VEGFR3 was regarded as a receptor with a minor function in Notch regulated angiogenesis. However, and in contrast to *Vegfc/Vegfd* double KO embryos that have no major defects in early blood vessel development, Vegfr3$^{-/-}$ embryos die at E10.5 with vascular defects[15], suggesting that VEGFR3 might also have an important but canonical ligand-independent function during angiogenesis that cannot be compensated by VEGFR2.

With this in mind we investigated in more detail how Notch regulates Vegfr3 function *in vivo*. In contrast with some previous reports we did not detect a substantial increase in *Vegfr3* transcription after impairing Notch signalling specifically in endothelial cells undergoing angiogenesis (Fig. 2a). But we did detect a strong upregulation of VEGFR3 protein levels and signalling activity in these cells suggesting that the regulation of VEGFR3 by Notch occurs mainly at the post-transcriptional levels (Fig. 2b). We also found that both Notch and VEGF regulate VEGFR3 levels in a opposite and independent manner. VEGF is normally required for VEGFR3 expression in angiogenic cells, but inhibition of Notch in vessels that lost VEGF activity rescues VEGFR3 levels (data not shown). To investigate the functional consequence of the high levels and activity of VEGFR3 protein in endothelial cells with low or compromised Notch signalling we used a kinase inhibitor (MAZ51) that potently inhibits VEGF-C- and VEGF-D-induced activation of VEGFR3 but only weakly impairs VEGFR2 activation by VEGF-A[16]. Although treatment of mice with MAZ51 alone, only weakly affected the number of filopodia and sprouts at the angiogenic front of the control retinal vasculature, this inhibitor strongly suppressed the enhanced sprouting caused by DAPT (Fig. 2c, d). These results showed that endothelial cells are normally not very sensitive to VEGFR3 kinase inhibition, whereas in the absence of Notch signalling VEGFR3 levels increase and in this context endothelial cells seem to be much more sensitive to VEGFR3 activity in accordance with previous results obtained in zebrafish where morpholinos or *Vegfr3* mutants were used[12,17].

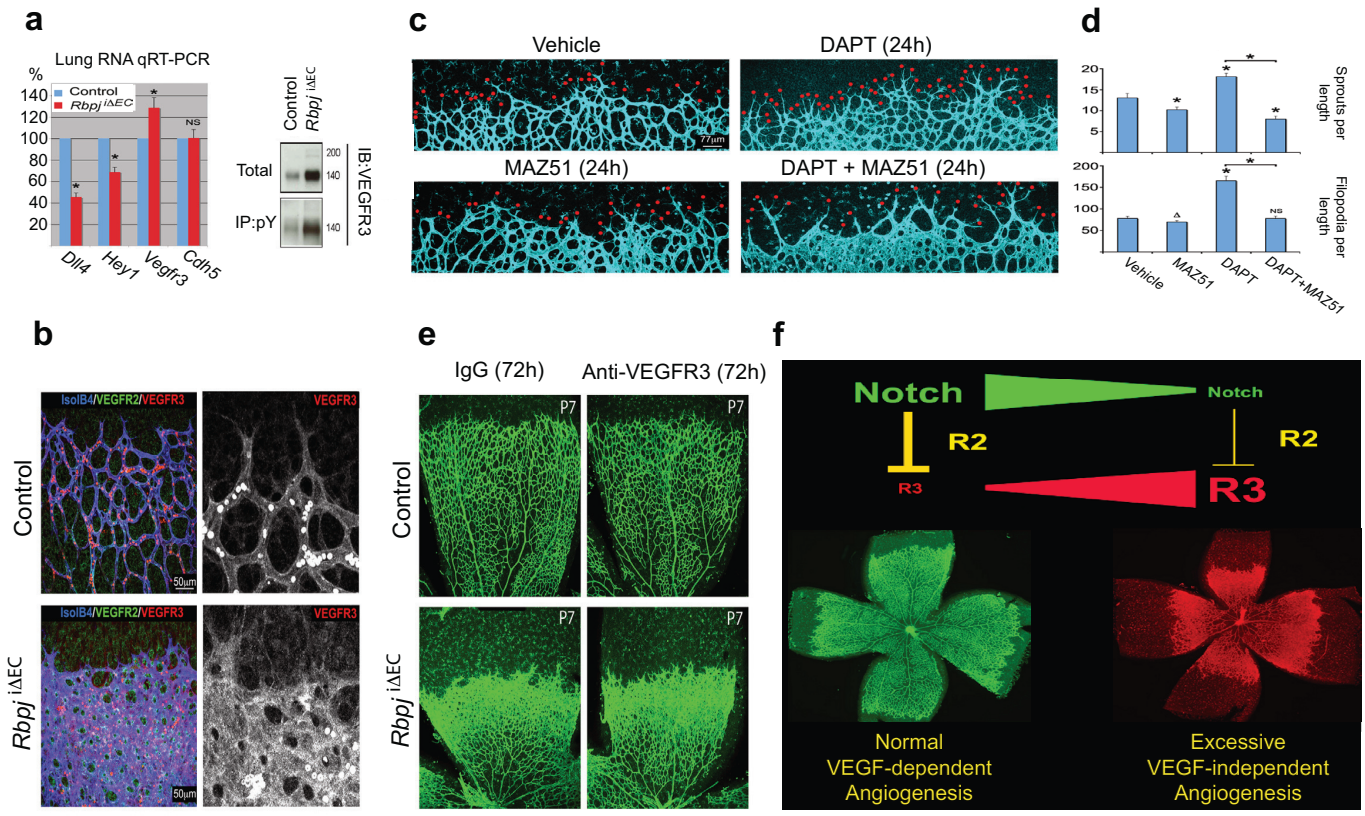

Figure 2
(a) qRT-PCR of *Rbpj*$^{i\Delta EC}$ post-natal day 6 (P6) mouse lungs for the indicated transcripts. On the right is shown the western blot result from lung lysates of the same animals, showing strongly increased total and phospho-VEGFR3 in *Rbpj*$^{i\Delta EC}$ mutants. Error bars represent s.e.m.; Asterisk, P < 0.001; NS, not statistically significant.
(b) Isolectin B4 (blue) combined with VEGFR2 (green) and VEGFR3 (red) staining of control and *Rbpj*$^{i\Delta EC}$ retinas. *Rbpj*$^{i\Delta EC}$ vessels have higher VEGFR3 protein levels. Bright, small and round cells are autofluorescent circulating blood cells.
(c, d) Angiogenic front vessels of a P6 mouse retina stained for isolectin B4 (blue) after 24 hrs of treatment (P5 to P6) with the indicated inhibitors. MAZ51 efficiently blocks sprouting (red dots) of DAPT-treated endothelial cells. Error bars represent s.e.m.; Asterisk, P < 0.05; NS, not statistically significant.
(e) Confocal images of isolectin B4–stained (green) control and *Rbpj*$^{i\Delta EC}$ retinas (tamoxifen administration from P1 to P3) of P7 mouse pups injected with control IgG or anti–VEGFR3 antibodies from P4 to P7 (72 hours).
(f) Schematic model illustrating the main findings. During angiogenesis, Notch activation in most endothelial cells downregulates VEGFR3, turning endothelial cell activity dependent on VEGF and VEGFR2 signalling. In endothelial cells with very low Notch signalling, VEGFR3 levels increase significantly and this leads to excessive and highly deregulated ligand-independent angiogenesis.

Altogether our results suggest that inhibition of Notch might switch blood vessel angiogenesis from a VEGF-A/VEGFR2 dependent mode to a VEGF-C/D/VEGFR3 regulated mode. To further test this hypothesis we used the blocking antibody mF4-31C1[18] to block binding of VEGF-C/D ligands to VEGFR3 during normal or Notch impaired angiogenesis. Contrary to the initial prediction, we did not see any significant difference in endothelial sprouting after the administration of this antibody in control or Notch mutant mice (Fig. 2e), suggesting that VEGFR3 might be active even in the absence of its canonical ligands as was also previously proposed[14,19,20]. Experiments performed by us with cell lines *in vitro* showed that the kinase inhibitor MAZ51 can block both ligand-dependent and independent VEGFR3 signalling, whereas the blocking antibody mF4-31C1 only blocks the ligand-induced signal (data not shown). We think that this difference explains the different *in vivo* effects of these two reagents and suggest that in the context of angiogenesis and low Notch signalling, VEGFR3 is active even in the absence of canonical ligand binding.

The sum of our findings indicate that during angiogenesis, all endothelial cells have Notch activity, although at relatively different levels, and in this context VEGFR3 levels are low and VEGF/VEGFR2 are the most important regulators of endothelial sprouting. In cells with very low Notch signalling or when we inhibit Notch, the situation changes, VEGFR3 protein levels increase significantly and it becomes phosphorylated even in the absence of the canonical ligands. We think that in this context there is ligand-independent and therefore highly deregulated endothelial sprouting (Fig. 2f), which mimics aspects of growth-

factor-independent (autocrine) cancer cell tyrosine kinase activity and growth[21]. This mode of abnormal vessel growth might contribute to the excessive and deregulated angiogenesis that is observed in tumors treated with Notch inhibitors[22,23]. Interestingly, it was previously shown that inhibition of Notch strongly impairs the growth of tumors, even the ones resistant to anti-VEGF[22], which is in agreement with our results and with the idea that Notch can control blood vessel biology in a VEGF-independent manner.

Anti-VEGF treatment of patients with cancer and age-related macular degeneration was already shown to significantly improve their condition. However many patients seem to respond poorly to anti-VEGF treatment and there are many cases of resistance[24,25]. According to our results probing the status of vascular Notch or VEGFR3 activation might be very relevant. This information could yield valuable clues for selecting suitable therapeutic strategies that could reduce the resistance to current anti-angiogenic therapies.

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Single-molecule reconstitution of actin regulatory mechanisms

Dennis Breitsprecher

The actin cytoskeleton is an inherent part of the eukaryotic cell. It not only provides mechanical support to maintain cell morphology, but also mediates many dynamic cellular processes. The rapid assembly and disassembly of filamentous actin arrays – e.g. during cytokinesis, vesicle trafficking, cell migration or adhesion - is regulated by the interplay of a large number of actin regulatory factors which affect all aspects of filament dynamics, ranging from nucleation and elongation over bundling and capping to filament severing and depolymerization.

A critical step at the onset of actin filament dynamics is the *de novo* formation of filaments, also called nucleation, which is mediated by nucleation factors (Chesarone & Goode, 2009). One family of nucleation factors, the formins, is particularly interesting. Formins not only promote the nucleation of new filaments, but also enhance their elongation rates by processively tracking the fast growing "barbed" end while inserting actin monomers (Breitsprecher & Goode, 2013; Kovar, 2006; Pruyne et al, 2002; Romero et al, 2004). Recently, *in vivo* and *in vitro* studies showed that collaborations between formins and other nucleation promoting factors (NPFs) are required in different organisms (Blanchoin & Michelot, 2012). However, the underlying molecular mechanisms are difficult to elucidate in bulk experiments due to overlapping functions of the proteins (e.g. nucleation of new filaments), and thus where elusive for a long time.

One elegant way to analyze such collaborations is to directly visualize the interactions of the assembly factors *in vitro* on the single molecule level. This can be achieved by multi-wavelength total-internal-reflection-fluorescence (TIRF) microscopy, which allows for the simultaneous imaging of multiple fluorescently tagged actin regulatory proteins and fluorescently labeled actin filaments (Figure 1A). Experiments with tagged variants of the formin mDia1 and the NPF adenomatous polyposis coli (APC) were instrumental to show that these proteins form dimer:dimer complexes which bind actin monomers to trigger efficient filament nucleation. After nucleation of the filament, mDia1 and APC complexes separate driven by filament growth, with the formin processively tracking the barbed end while APC stays bound the site of nucleation (Figure 1A and B) (Breitsprecher et al, 2012). Further experiments showed that this mechanism was required for filament formation in the presence of actin-assembly inhibitors, such as the sequestering protein profilin and barbed end capping proteins. This example highlights the power of single-molecule imaging to dissect complex multi-component mechanisms in a dynamic system.

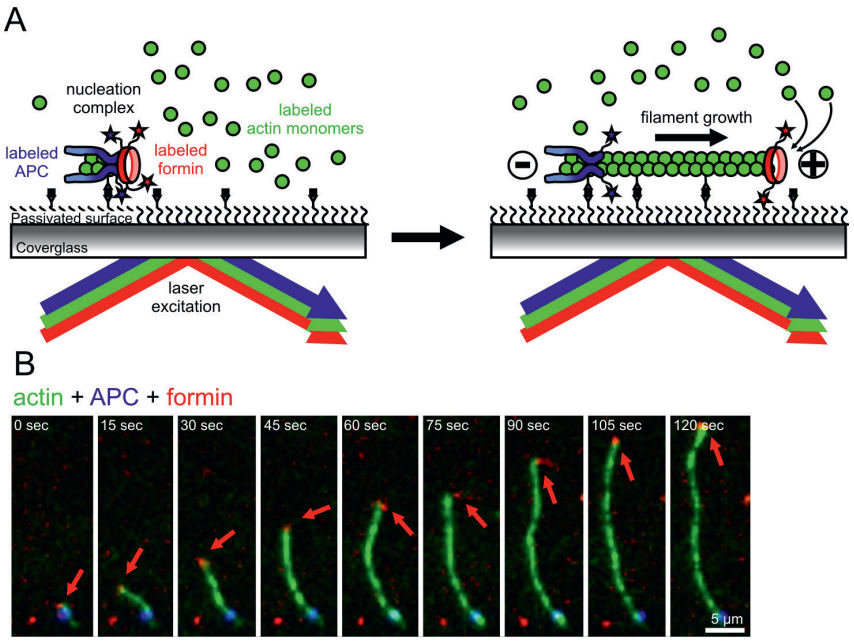

Figure 1
Single molecule imaging of complex actin assembly mechanisms. A) Schematic representation of APC/mDia1 collaboration. An actin filament is nucleated by the APC/mDia1 nucleation complex and anchored to a passivated glass surface, with APC residing at the pointed end (-) while mDia1 elongates the barbed end (+). B) Single-molecule TIRF micrographs of an actin filament nucleated by APC-mDia1 collaboration and subsequently elongated by mDia1 (red arrow).

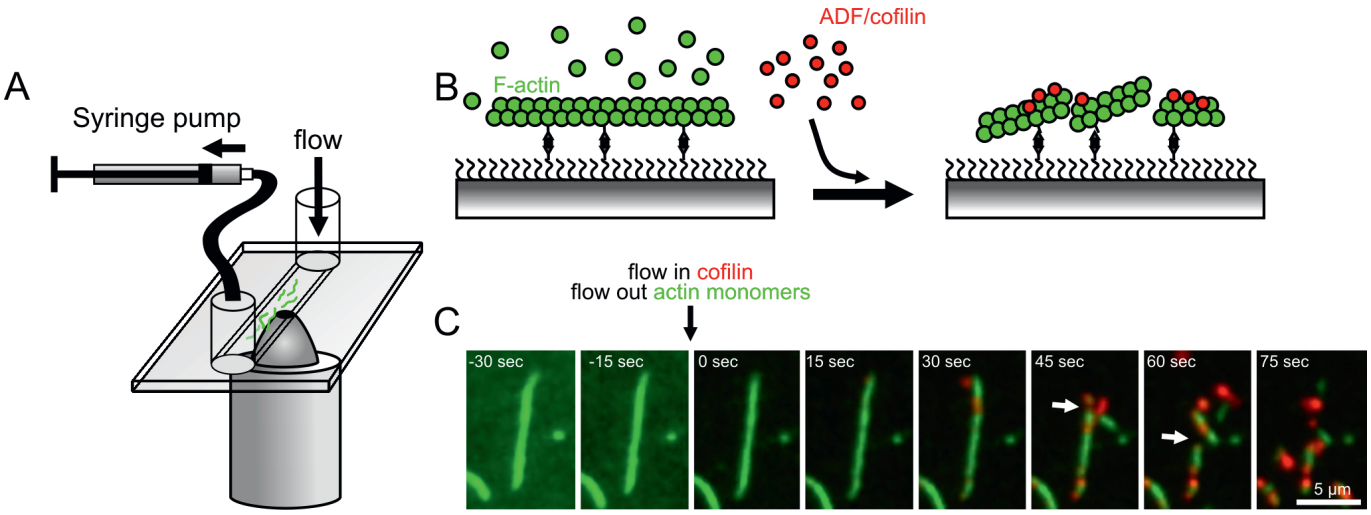

Figure 2
Reconstitution of actin disassembly. A) Schematic representation of a microfluidic chamber on an inverted TIRF-microscope objective. Flow can be induced by pulling liquid through the flow chamber. B) Schematic representation of F-actin disassembly after addition of ADF/cofilin. C) TIRF micrographs of actin filament disassembly by fluorescently tagged cofilin (red). Cofilin is added at time 0, and the binding of cofilin and subsequent severing of filaments (arrows) can be observed over time.

In addition, single-molecule approaches are very versatile, and can also be used to elucidate mechanisms of filament disassembly and turnover, with just some minor modifications of the experimental design. The example in Figure 2A shows a TIRF setup with a microfluidic flow chamber. Actin filaments are first polymerized in the flow chamber, and then monomers are washed out and fluorescently tagged disassembly factors are added to trigger depolymerization (Figure 2B). The major actin filament disassembly factor in cells is the small F-actin binding protein cofilin. By using fluorescently tagged cofilin molecules in microfluidic experiments, accumulation of cofilin in distinct patches along the length of the actin filament can be observed during depolymerization. Cofilin binding eventually induces filament breaks, leading to filament fragmentation (Figure 2C). These experiments showed that a cofilin-binding protein, the ubiquitous cyclase-associated protein (Srv2/CAP), specifically enhances cofilin severing efficiency, and that this activity was required for filament disassembly and turnover in cells (Chaudhry et al, 2013). Thus, filament disassembly processes, just as filament assembly processes, are controlled by interactions of their regulatory factors, and not by the activity of one single protein. We are just beginning to understand the complex mechanisms that regulate highly diverse actin-dependent processes in the cell. In the future, single-molecule reconstitution approaches will likely play a major role in gaining a comprehensive systems understanding of how the actin cytoskeleton is regulated on the molecular level (Mullins & Hansen, 2013).

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Need for speed: Tracing chromatin remodelers in search of the right nucleosome

Fabian Erdel

In eukaryotic cells, genomic DNA is packaged into a complex with histone proteins, which is called chromatin. A large portion of the DNA is tightly wrapped around histone octamers to form nucleosomes, which restrict the access to the underlying sequence information. Thus, the positioning of nucleosomes is an important determinant for the accessibility and functionality of the genome. To actively control nucleosome positions, cells utilize chromatin remodelers that can translocate or remove nucleosomes upon ATP hydrolysis (Erdel et al., 2011a). Although these enzymes have been studied extensively *in vitro*, their behavior in the context of the physiological chromatin template remains poorly understood. In particular, it is elusive which nucleosome positions are regulated by which remodeling enzyme, what is the targeting mechanism that renders a nucleosome a substrate, and how frequently nucleosome translocations occur. To address such questions, it is instructive to study GFP-tagged remodelers in living cells by fluorescence microscopy. In particular, fluorescence fluctuation microscopy allows not only to visualize the localization of the enzymes but provides additional information about their mobility and the interactions they undergo.

There are several techniques to study the mobility of fluorescent proteins in living cells (Erdel et al., 2011b). Most of them are related to Fluorescence Recovery After Photobleaching (FRAP) or Fluorescence Correlation Spectroscopy (FCS). In FRAP, a region of interest is bleached with high laser intensity, and subsequently an image series is acquired that captures the motion of the bleached particles out of the bleach region (Fig. 1A). Based on this image series, the diffusion coefficient

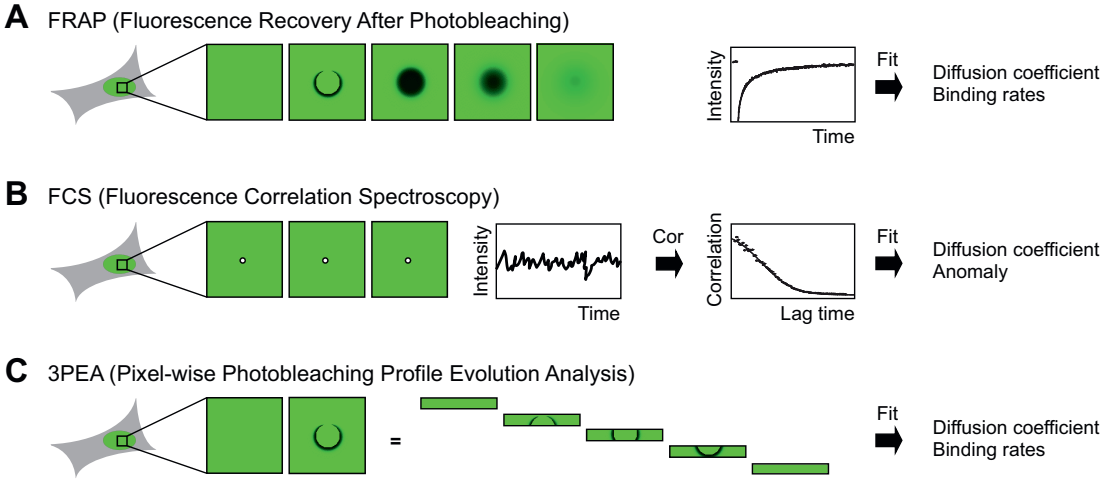

Figure 1 Fluorescence fluctuation microscopy techniques. Different techniques can be used to measure the mobility of fluorescently tagged proteins in living cells. (A) In Fluorescence Recovery After Photobleaching (FRAP), particles within a region of interest are bleached, and subsequently the intensity in the bleach spot is recorded over time. The shape of the recovery curve encodes information about the diffusion coefficient of the protein and the kinetic rate constants for the binding interactions it undergoes. (B) In Fluorescence Correlation Spectroscopy (FCS), the focal volume of a confocal microscope is parked at a fixed position, and the intensity is measured over time. When particles enter and leave the observation volume the intensity fluctuates, and the properties of these fluctuations can easily be analyzed after calculating the autocorrelation function. Fitting of this function yields the diffusion coefficient and the anomaly parameter that characterizes the structural complexity of the cellular environment. (C) In Pixel-wise Photobleaching Profile Evolution Analysis (3PEA) particles within a region of interest are bleached, and the spatiotemporal distribution of bleached particles that move during the bleach process is fitted. This yields information about the diffusion coefficient and the rapid binding interactions of the protein under study.

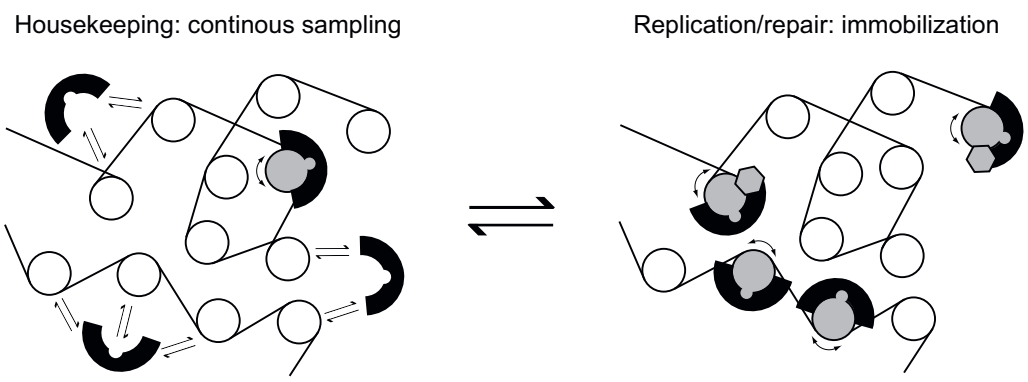

Figure 2 Continuous sampling model for ISWI chromatin remodelers.
ISWI chromatin remodelers bind nucleosomes transiently to identify appropriate substrates. Under "housekeeping" conditions, nucleosomes are translocated rather rarely. Only in special cases such as DNA replication or repair, ISWI remodelers bind tightly to chromatin and become active.

of the protein and the kinetic rate constants for binding to immobile obstacles can be obtained. In FCS, the focus of a confocal microscope is placed at a fixed position, and fluorescent molecules that move in or out of the observation volume are detected (Fig. 1B). From the recorded intensity fluctuations the diffusion coefficient of the particles is obtained. Both groups of methods provide complementary information: FRAP is typically suited for processes that occur within seconds to minutes, whereas FCS has high time resolution down to microseconds but does not work for slower processes due to limited photostability of the available fluorophores. In the range between hundreds of milliseconds to a few seconds, both FRAP and FCS have difficulties to reliably resolve molecular mobility and interactions. Since the catalytic rates measured for chromatin remodelers *in vitro* suggest that remodeling can occur on this timescale (Blosser et al., 2009; He et al., 2006), I devised a method to assess processes on these scales, which we termed Pixel-wise Photobleaching Profile Evolution Analysis (3PEA). It relies on fitting the intensity distribution around the bleach region (Fig. 1C). Using FRAP, FCS and 3PEA I studied the mobility of the human Imitation Switch (ISWI)-type chromatin remodelers Snf2H and Snf2L in living cells (Erdel and Rippe, 2012; Erdel et al., 2010). Interestingly, ISWI remodelers are very mobile, bind only transiently to chromatin and seem to translocate nucleosomes rather rarely. Only in special cases, e.g. at replication foci or at DNA damage sites, many remodelers bind long enough to translocate nucleosomes (Fig. 2). Given the moderate activity of remodelers within their cellular environment it is surprising that they are expressed at relatively high levels in the micromolar range as measured by FCS and quantitative western blotting. This apparent contradiction might be resolved when the frequency at which a nucleosome is visited by a remodeler is calculated based on the values obtained from fluctuation microscopy. To quickly react upon external stimuli such as occurrence of DNA damage, a high frequency is required, which can be accomplished by high remodeler abundance and mobility. Indeed, ISWI proteins accumulate at laser-induced DNA damage sites within several seconds to a few minutes (Erdel and Rippe, 2011), which would not be possible with lower concentrations. Thus, the cell seems to fine-tune its remodeling machinery to ensure sufficient chromatin plasticity.

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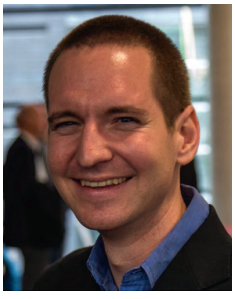

Fabian Erdel studied Physics and Molecular Biology at Ruprecht-Karls-University in Heidelberg, Germany, and at Tor Vergata University in Rome, Italy. For his PhD he joined the lab of Karsten Rippe at the German Cancer Research Center (DKFZ) and BioQuant Center in Heidelberg, where he applied fluorescence microscopy based methods to study interactions of chromatin remodelers in living cells. Currently, he is working as a postdoc in Karsten Rippe's lab with a focus on light-induced manipulation of activities in the cell nucleus.

Towards a better understanding of actin dynamics in vivo – a high resolution view of the fly actin cytoskeleton

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Introduction

The actin cytoskeleton provides mechanical forces to drive cell shape changes and cell migration during morphogenesis. For initiation of actin polymerization, a nucleation core (nucleus) composed of three actin monomers is required. Once this actin nucleus is formed, actin polymerizes spontaneously into long filaments (Figure 1). In vivo, the actin polymerization is strictly controlled in time and space. Eukaryotic cells have evolved a multitude of actin binding proteins that maintain the pool of actin monomers, promote actin nucleation, restrict the length of actin filaments and cross-link filaments into networks or bundles (Figure 1; Pollard & Borisy, 2003, Pollard & Cooper, 2009). Thus, these accessory proteins also define the mechanical and dynamic properties of actin filaments and differentially control the organization of actin filaments into higher-order structures adapted to fulfill distinct cellular functions. New superresolution microscopy techniques such structured illumination microscopy (SIM) allow to visualize actin structures as well as protein localization at high spatial and temporal resolution, a perquisite for a better mechanistic understanding how actin regulatory proteins act in concert in vivo.

Actin nucleation – a key step in actin polymerization

The rate-limiting step in actin polymerization is the de novo nucleation of actin filaments from actin monomers. To overcome this high kinetic barrier, cells have evolved a variety of actin nucleators that catalyze the nucleation reaction. The Arp2/3 protein complex is one of the key players initiating de novo for-

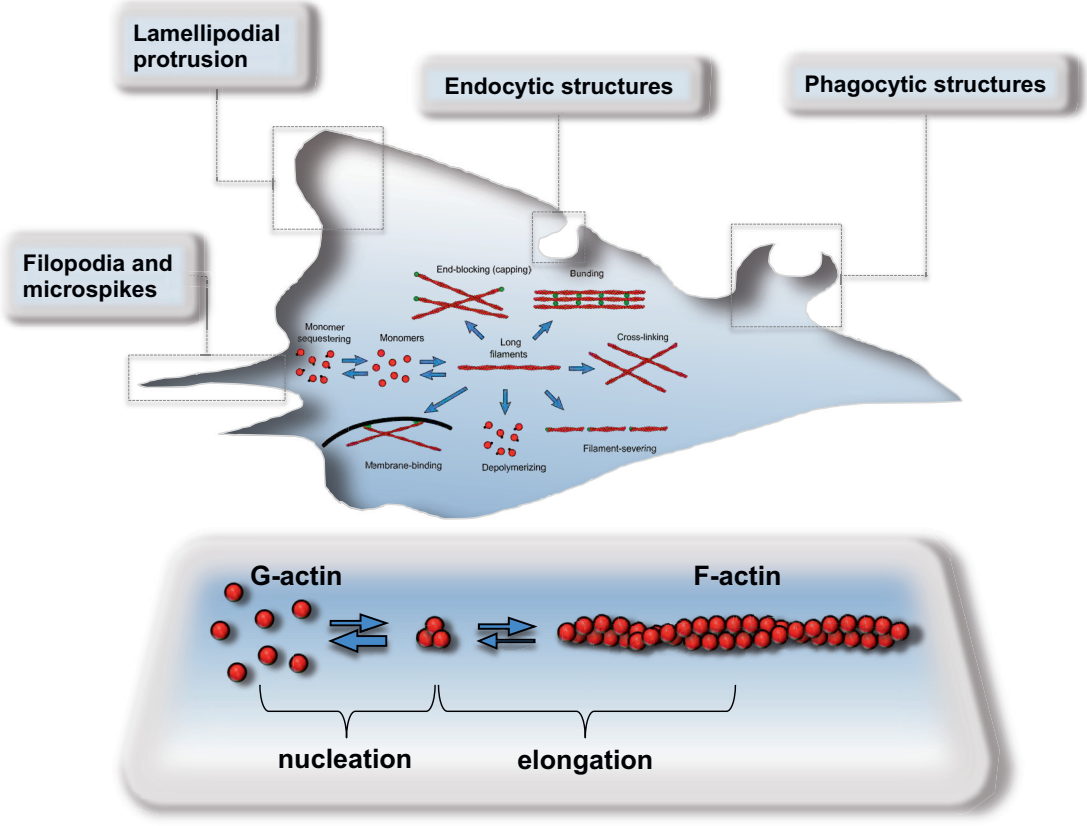

Figure 1: Actin binding proteins controlling distinct aspects of actin cytoskeleton architecture and dynamics. Schematic drawing of an eukaryotic cell, depicting the different cellular roles of actin binding proteins in lamellipodial and filopodial protrusion, endocytosis and phagocytosis (adapted from Lodish et al., Molecular Cell Biology, 6th ed., 2008). For initiation of actin polymerization, a nucleation core (nucleus) composed of three actin monomers is required. Once the actin nucleus is formed, additional actin monomers bind to the nucleus spontaneously resulting into a fast filament elongation.

mation of actin filaments. Compared to other so far identified actin nucleators such as Formins or Spire the the Arp2/3 complex mainly initiates actin filaments on the sides of preexisting mother filaments resulting in branched networks of actin filaments enriched near the leading edge of cells (for review: (Goode and Eck, 2007; Chesarone and Goode, 2009). This coupling of actin nucleation and filament branching establishes the basis of the dendritic nucleation model, which implies repeated cycles of branching nucleation of actin filaments by the Arp2/3 complex generating the forces to push cell membranes (Mullins et al., 1998). Improved cyro-electron tomographs confirmed the existence of branched actin filaments, however these filaments of variable length are not concentrated at the front but rather distributed throughout protruding lamellipodia (Vinzenz et al., 2012). Thus, the authors proposed that branching might be important for generating an actin network, but force generation is not dependent on short filaments generated at branch points (Vinzenz et al., 2012, Small et al., 2008). This also implies a new model for membrane protrusion that requires an unknown crosstalk between different actin nucleating, elongating and crosslinking complexes in vivo.

Wiskott-Aldrich syndrome protein (WASP) family members – key regulators of the actin cytoskeleton

The activity of the Arp2/3 nucleation machine is controlled by so-called nucleation promoting factors (NPF) such as members of the Wiskott-Aldrich syndrome protein (WASP) family that drive actin polymerization in time and space (Derivery and Gautreau). In vertebrates, the WASP/WAVE protein family consists of eight different proteins: the two Wiskott-Aldrich syndrome proteins WASP and N-WASP, the related WASP family Verprolin homologous proteins WAVE 1-3 (also called SCAR 1-3), the Wiskott-Aldrich syndrome protein and SCAR Homolog WASH (Derry et al., 1994; Miki et al., 1996; Miki et al., 1998; Symons et al., 1996; Linardopoulou et al., 2007; Liu et al., 2009) and the recently identified the WASP homolog associated with actin, membranes and microtubules WHAMM and the Junction mediating and regulatory protein JMY (Campellone et al., 2008; Zuchero et al., 2009).

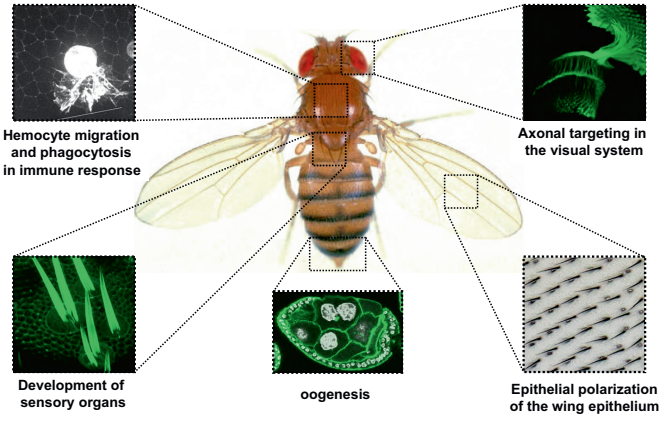

Figure 2: Actin driven processes in Drosophila development.
Despite their similar biochemical properties WASP proteins fulfill distinct cellular functions in vivo during Drosophila development. For references see text.

WASP proteins possess a common C-terminal WCA domain, which is required and sufficient to activate the Arp2/3 complex (Rohatgi et al., 1999), whereas the amino-terminal and central regions of these proteins show a remarkable divergence providing significant differences in their activity and regulation. WASP proteins are regulated by similar molecular principles. The activities of WASP, WAVE and WASH are controlled by multiprotein complexes regulating the localization, the stability and the activity (Campellone and Welch, 2011; Rottner and Stradal, 2011; Insall and Machesky, 2009; Pollitt and Insall, 2009). Under resting conditions the NPFs are primarily inactive and become activated upon binding of the Rho GTPases such as Cdc42 and Rac1, phosphorylation or lipid binding.

In contrast to vertebrates, Drosophila has only single gene copies of wave, wasp and wash, thus analyses are not complicated by redundancy (Ben-Yaacov et al., 2001; Zallen et al., 2002; Liu et al., 2009). The phenotypic analysis of the fly mutants also revealed that WAVE, WASP and WASH have some overlapping functions but rather differentially regulate distinct aspects of Arp2/3 activity during development such as hemocyte motility, oogenesis, wing morphogenesis, photoreceptor axon targeting or sensory organ formation (Figure 2; Zallen et al., 2002; Gohl et al., 2010; Stephan et al., 2011; Ben-Yaacov et al., 2001; Bogdan and Klämbt, 2003; Bogdan et al., 2004; Bogdan et al., 2005; Leibfried et al., 2008; Stephan et al., 2008; Fricke et al., 2009; Yan et al., 2013; Zobel and Bogdan, 2013).

How do WASP proteins regulate distinct aspects of Arp2/3 dependent cellular and developmental functions? Among all WASP protein family members, the cellular function and the molecular regulation of the WAVE/SCAR proteins are best understood. WAVE is trans-inhibited in a heteropentameric protein complex (WRC, WAVE regulatory complex) with the Abelson interactor (Abi), Nap1/Kette, specifically Rac-1 associated protein 1 (Sra-1) and hematopoietic stem progenitor cell 300 (HSPC300) (Eden et al., 2002; Bogdan and Klambt, 2003; Derivery et al., 2009; Lebensohn and Kirschner, 2009). WAVE stability depends on the integrity of the complex and coinciding signals such as activated Rac. The catalytic VCA motif of WAVE is sequestered by a combination of intramolecular and intermolecular contacts within the WAVE complex (Chen et al., 2010). Upon Rac1 binding to Sra-1 the VCA domain is released and WAVE becomes active. This model also implies that acidic phospholipids cooperate with Rac1 to recruit the complex to the membrane by binding to the positively charged faces of the Sra-1/Nap1/Kette platform and the polybasic region of WAVE. Since only half of the Abi protein lacking the proline-rich regions and the SH3 domain was struc-

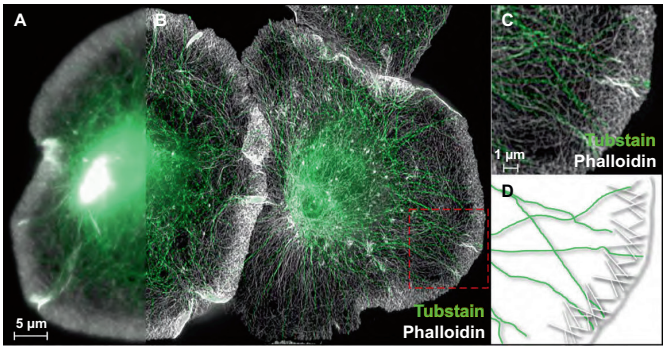

Figure 3: The actin and the microtubule cytoskeleton of Drosophila S2R+ cells.
Maximum intensity projection image of S2R+ cells stained with phalloidin-Alexa488 (F-actin) and Tubstain-TexasRed (microtubules). (A) a part of the entire image visualized by conventional wide-field microscopy and (B) imaged by structured illumination microscopy (SIM). The dense zone of actin network at the periphery corresponds to the lamellipodium. (C) A subset of the lamellipodium is enlarged from image B. Scale bars are shown. (D) Schematic drawing of the branched actin network (white-grey) at the lamellipodium tip and single microtubules (green). Images taken from Zobel and Bogdan, 2013.

turally analyzed, the exact molecular function of Abi within the WAVE complex remains still unclear (Chen et al., 2010).

The cytoskeleton of fixed Drosophila cells imaged by structured-illumination microscopy (SIM)

A better mechanistic understanding of the cellular and developmental functions of WASP proteins requires new microscopy techniques which allow to visualize and compare protein localization as well as actin structures at high spatial and temporal resolution in different genetic backgrounds. Among these approaches is the structured illumination microscopy (SIM), a wide-field technique that doubles both lateral (100nm) and axial (250nm) resolution (Gustafsson et al., 2008). Compared to the conventional wide-field microscopy images, SIM strikingly improved the resolution of cytoskeletal structures of cells (Figure 3). Single microtubules and filamentous actin within the dense actin network are clearly better resolved at the cell periphery of Drosophila S2R+ cells, hemocyte-like cells originally derived from late stage Drosophila embryos (Figure 3A, B).

Endogenous WAVE/SCAR strongly localizes at the tip of lamellipodia of S2R+ cells where it drives membrane protrusions (Figure 4A, B; Rogers et al., 2003, Bogdan et al., 2005). Loss of wave function causes a complete disruption of lamellipodia. RNAi mediated knock down of Arp2/3 or WRC function result in a characteristic starfish-like cell morphology with multiple filopodia-like cell extensions (Rogers et al., 2003, Kunda et al., 2003, Bogdan & Klämbt, 2003). Recent SIM analysis of WAVE-depleted S2R+ cells document the dramatic reorganization of the actin cytoskeleton (Zobel and Bogdan, 2013; Figure 4C, D). The dense actin meshwork of lamellipodia is completely disrupted. Knock-down cells show a loosely packed actin network and thick actin bundles extending into the filopodia-like structures.

Visualization of higher-order actin structures in Drosophila at high spatial resolution

The combination of super-resolution 3D microscopy with Drosophila genetics and cell biology further allows to get detailed insights into the structural and molecular requirements of different actin-dependent processes in vivo, in a multicellular environment. Drosophila oogenesis provides an excellent genetic model system, which requires a stereotypic set of actin-dependent cellular processes including intercellular transport, stable intercellular bridges, collective cell migration and cell shape changes (Hudson & Cooley, 2002a, Bastock & St Johnston, 2008, He et al., 2011). Drosophila ovaries contain progressively maturing egg chambers. Each of them are composed of a polarized epithelium of follicle cells surrounding 15 nurse cells and one oocyte that are connected by F-actin rich cytoplasmic bridges (ring canals, see Fig. 4). Recent data demonstrate that 3D SIM can have a major advantage over conventional confocal imaging of Drosophila egg chambers (Zobel and Bogdan, 2013). Highly patterned three-dimensional actin structures such as ring canals or cytoplasmic bundles of actin filaments are clearly better resolved (Figure 5). SIM analysis also revealed a more loosely packed actin network (average size between filamentous

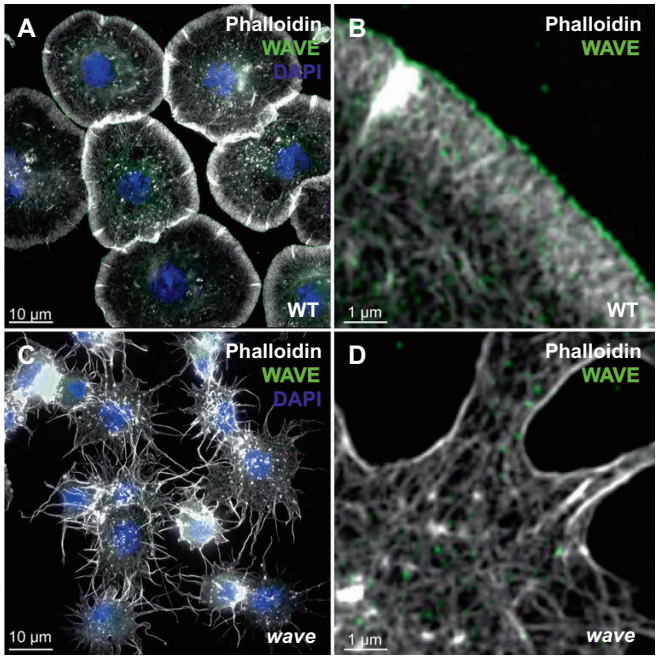

Figure 4: Disruption of the lamellipodial actin meshwork in wave depleted S2R+ cells.
Maximum intensity projection images of wild type S2R+ cells (A, B) and wave depleted S2R+ cells (C, D) stained with phalloidin-Alexa488 (F-actin, white), anti-WAVE (green) and DAPI to visualize nuclei (blue). (B, D) Subsets of the cell periphery are enlarged from images A and C, respectively. Images taken from Zobel and Bogdan, 2013.

actin 190 nm) forming the ring canal as previously observed using electron microscopy (Tilney et al., 1996). Thicker actin filament bundles (> 229 nm) form a basket around the ring canals and thinner cytoplasmic actin filament bundles (103-230 nm; measurements in figure 5D) extend from the cortex to the nuclei. Loss of of the Arp2/3 or WRC function results in smaller and abnormally shaped eggs (Zallen et al., 2002, Hudson & Cooley, 2002a). Similar phenotypes are observed in flies lacking maternal Abi protein, an integral subunit of the WAVE complex. Ring canals of abi mutant egg chambers are smaller than in wild-type and often aberrantly shaped (Zobel and Bogdan, 2013). Like in later stage scar mutant egg chambers, ring canals are often detached from the nurse cell membranes (Figure 5G-I, arrows) and the subcortical actin becomes destabilized resulting in multinucleated cells in abi mutants. Cytoplasmic bundles are still present in mutant nurse cells, although they are highly disorganized probably due to membrane destabilization (Fig. 4 G). Thus, WAVE-Arp2/3 mediated actin polymerization does not seem necessary for the initial formation of these actin structures but rather important for their maintenance and for membrane integrity.

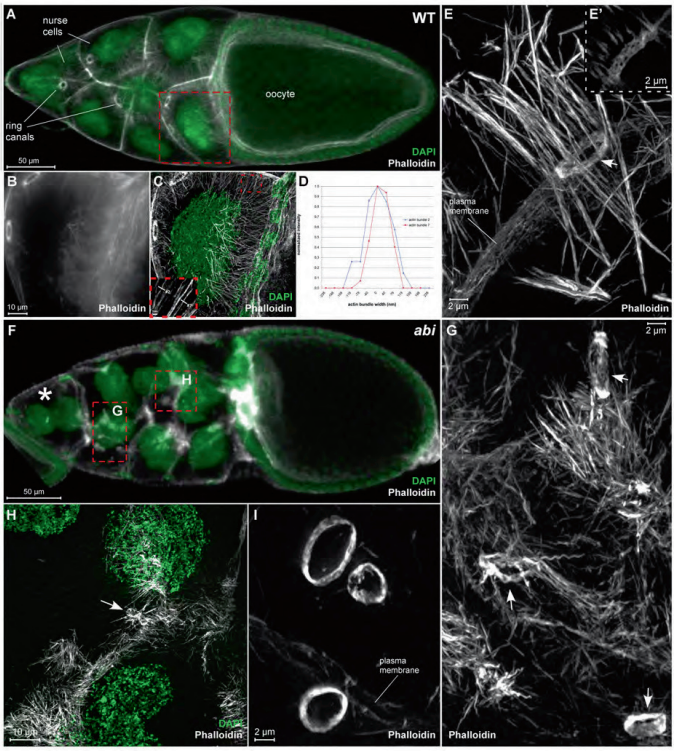

Figure 5: Fig. 5. 3D-SIM imaging of actin structures in Drosophila egg chambers
(A) Conventional confocal image (LSM 510, Zeiss) of an early wild type Drosophila stage 10B egg chamber stained with Alexa488 phalloidin to reveal actin structures (white), and DAPI to visualize nuclei (green). Actin structures such as ring canals, polyploid nurse cells and epithelial follicle cells surrounding the oocytes are apparent. (B) Maximal intensity projection of a conventional confocal image (LSM 510, Zeiss), (C) a 3D-SIM image of the boxed region (red) in A. Cytoplasmic actin filament bundles extend from the nurse cell cortex. (D) Plots of intensity along respective lines (#2 and #7) in C. The apparent full width at half maximum (FWHM) was measured. (E) 3D-SIM reconstruction of a ring canal (arrow) surrounded by a basket of actin filament bundles. (E') Single image of the 3D-SIM reconstruction in (E) showing loosely packed filamentous actin. (F) Conventional confocal image (LSM 510, Zeiss) of an abi mutant 10B egg chamber stained with phalloidin (white) and DAPI (green). Subcortical F-actin became destabilized resulting in multinucleated nurse cells (asteriks), (G, H) 3D-SIM images of the boxed regions (red) in F. Ring canals of mutant egg chambers are often aberrantly shaped (arrows in G, H), while cytoplasmic bundles are highly disorganized probably due to membrane destabilization. (I) 3D-SIM image of ring canals detached from the membrane. Scale bars are shown. Images taken from Zobel and Bogdan, 2013.

Concluding remarks

The enhanced resolution of the SIM technique now opens up the perspective to revisit "old friends" as well as to identify new candidates controlling the organization of the actin cytoskeleton at the single cellular and multicellular level. Genetically traceable model systems such as Drosophila egg chambers or macrophages (called hemocytes) further allow researchers to combine genetics with new advanced high resolution microscopy techniques and live cell imaging in order to identify and characterize the conserved regulatory network controlling cell shape and cell motility in vivo.

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Forcing random cell movements into a functional form: The cellular choreography of cardiac morphogenesis in fish

Salim Abdelilah-Seyfried

Introduction

The first historical report of an inversely positioned heart dates back to the 15th century when the omnitalented "Renaissance man" Leonardo da Vinci artistically recorded this morphological rarity. His observation highlights the profound surprise that such an unusual positioning of this organ elicited at that time. In most of us, visceral organs such as heart, lung, liver, gut, and spleen are stereotypically distributed within the body cavity with the heart on the left side. Not only the position but also the morphology of the heart is highly asymmetric with respect to the left/right (L/R) axis [one of the three body axes in vertebrates, alongside the anterior-posterior (head-to-toe) and the dorsoventral (back-to-front) axes]. To a large extent this morphological asymmetry serves cardiac function. The left ventricular chamber is larger and thicker and supports the main systemic circulatory system, whereas the smaller right ventricular chamber sustains only the smaller circulatory system of the lungs. Similarly, the positions and distribution of major arteries and the two atrial chambers is highly asymmetric with respect to the L/R axis. An abnormal morphogenesis or mispositioning of the heart endangers its efficiency; during an average human lifetime, the heart performs a truly Herculean and relentless task: beating approximately some 2.5 billion times – and pumping an estimated 190 million liters of blood through the vascular network to support all bodily functions.

Today studies in zebrafish and other model organisms have shown us that this highly stereotypical L/R asymmetry of heart morphology is the result of invariant asymmetrical development. Two principal signaling cascades that have been conserved through evolution - Nodal and the Bone morphogenetic protein (Bmp) signaling pathways - are essential in establishing cardiac laterality (Zhang and Bradley, 1996; Schilling et al., 1999; Breckenridge et al., 2001; Chocron et al., 2007; Smith et al., 2008; Schier et al., 2009; Chen et al., 2010). Both Nodals and Bmp molecules belong to the larger transforming growth factor ß (TGFß) superfamily of signaling molecules. These factors function by binding to their respective type I and type II serine/threonine kinase receptors, which, upon ligand binding, phosphorylate a set of downstream receptor-regulated-Smad transcription factors. Although Nodals and Bmps bind to different receptor complexes and pass signals via different groups of Smad proteins, both signaling cascades require the association of the phosphorylated (p)Smads, with a common co-Smad4 for nuclear translocation and target gene activation. Until recently, both the potential mode of interaction between Nodals and Bmp and the target genes activated by these two TGFß signaling pathways during heart development were unknown.

Over the past two decades, zebrafish (*Danio rerio*) has become the model organism of choice to elucidate the cellular behaviors and molecular pathways that underlie early heart development (Staudt and Stainier, 2012). Several impressive features contribute to the popularity of this organism for such analyses. First, zebrafish embryonic development can proceed even in the absence of a functional heart because the embryos are so small that nutrients and oxygen can freely diffuse into the embryo proper and sustain embryonic life for several days on their own. Second, the zebrafish embryo is transparent and has an extra-uterine mode of development. This has allowed powerful live imaging approaches, particularly in transgenic embryos that carry tissue- or cell type-specific transgenic reporter constructs. Finally, the feasibility of modifying gene functions through genetic gain- and loss-of function approaches makes this organism a prime candidate for the analysis of developmental factors, including genes that potentially play a role in human cardiac diseases. These properties have permitted the collection of a larger number of mutants with defective cardiac morphogenesis and an analysis of the respective loss-of-function effects of candidate genes: the misguided behavior of cardiac progenitor cells can be followed via four-dimensional high-resolution *in vivo* microscopy. Their analysis has led to surprising functional insights into the morphogenesis of the heart.

Heart tube formation is the result of a process of epithelial morphogenetic transformation

Zebrafish cardiac development results from complex cell rearrangements involving myocardial and endocardial progenitor cells, the two main types of cells that form the nascent heart tube (reviewed in Staudt and Stainier, 2012). Myocardial cells produce the outer muscular layer of the heart whereas endocardial progenitor cells give rise to the specialized endothelial

internal lining of the heart tube. Both cell types are derived from two bilateral fields of lateral plate mesoderm, from which they first initiate symmetrical movements towards the embryonic midline (Trinh et al., 2004; Bussmann et al., 2007; Holtzmann et al., 2007). In a second step, the cardiac cone reshapes and breaks midline symmetry by typically extending towards the left. This process, known as cardiac jogging, produces not only L/R asymmetry but also results in the transformation of the flat cardiac cone into an extending, hollow heart tube (Chen et al., 1997; Rohr et al., 2008; Fig. 1).

The complexity of the morphogenetic rearrangement is apparent when one considers the challenge of transforming a flat epithelial sheet that is tightly constrained by neighboring tissues into an elongated heart tube. The process depends on rapidly changing and dynamic cellular behaviors. Within the heart cone, myocardial cells exhibit strikingly different shapes depending on their positions. Cells at the center, close to the midline, exhibit columnar shapes, whereas cells at more lateral positions are cuboidal or even squamous (Trinh and Stainier, 2004). Hence, myocardial cells apparently undergo a process of epithelial maturation throughout the cardiac field. In support of this observation, the loss of atypical protein kinase C iota (aPKCi) or of Membrane protein palmitoylated 5 (Mpp5), two components of the Partition (Par) and Crumbs protein complexes of cell polarity regulators, completely abolishes heart tube formation (Horne-Badovinac et al., 2001; Peterson et al., 2001; Rohr et al., 2006; Rohr et al., 2008). Thus, early heart tube formation is an epithelial tissue transformation process that requires that cardiac progenitor cells must be organized as a highly polarized epithelial tissue.

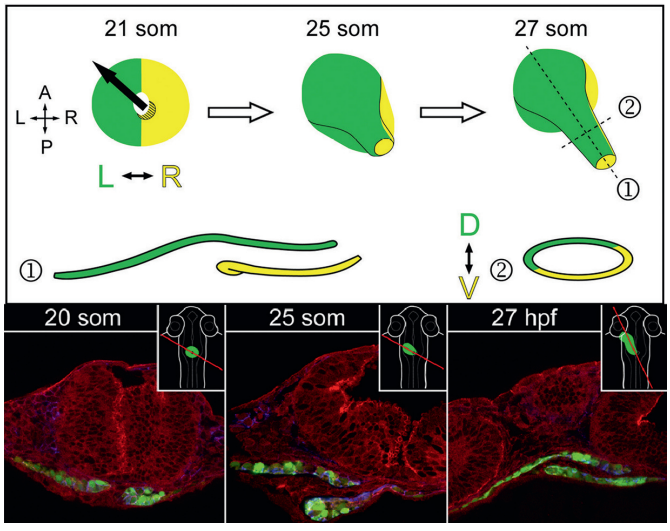

Figure 1:
Top row shows a schematic representation of the heart cone-to-tube transition. The left side of the cardiac cone (green) contributes to the dorsal roof whereas the right side (yellow) will form the ventral floor of the nascent heart tube. The dorsoventral axis of the heart tube at the 27-somite (som) stage is depicted in longitudinal (1) and transverse sections (2). Below, cross sections through the heart field in myocardial reporter Tg(*myl7:EGFP*)^{twu34} transgenic embryos (myocardial cells marked green; F-actin, red) show the progressive formation of the nascent heart tube (Rohr et al., 2008).

Cardiac tube formation commences with the occurrence of an involution fold of myocardial cells that are within the right half of the cardiac cone and move ventrally to form the ventral floor. Left-sided myocardial cells, on the other hand, do not involute; instead they establish the dorsal roof of the nascent heart tube. This intricate process generates the first morphological asymmetry in the zebrafish embryo and progressively transforms the flat cardiac cone into the heart tube (Rohr et al., 2008; Fig. 1). By the end of the transformation process, endocardial cells that were initially positioned below the cardiac cone are included within the heart tube (Bussmann et al., 2007). Live confocal microscopy revealed that left-sided myocardial and endocardial cells move with higher velocities than those on the right (Baker et al., 2008; de Campos-Baptista et al., 2008; Rohr et al., 2008; Smith et al., 2008; Lenhart et al., 2013; Veerkamp et al., 2013). The fact that L/R asymmetry of the heart depends on the Nodal co-receptor One-eyed pinhead, left-sided Nodal ligand Southpaw (Spaw)/Nodal-related 3 (Ndr3)(which is referred to as Spaw), and Bmps raised the intriguing possibility that the L/R asymmetric behavior of cardiac cells may be under the direct control of Nodal and Bmp signaling. The functional relationship of Nodals and Bmps, the consequences of complex TGFß signaling for cellular behaviors, and the target genes involved in the execution of this process were largely unknown and have become the focus of intensive studies.

Nodal negatively modulates Bmp activity by unilaterally biasing the extracellular matrix composition

Among the great unresolved questions of L/R asymmetry are the mechanisms by which Nodal signaling, once established, influences the cellular behaviors that underlie heart morphogenesis (or, for that matter, morphogenesis of other midline organs such as the gut). A number of excellent studies have reviewed the mechanisms by which a L/R asymmetry of Nodal signaling is initiated in vertebrates and will not be discussed here. In the zebrafish embryo, the L/R asymmetry of visceral organs depends on the Nodal ligand Spaw, one of three Nodal ligands present in that organism (Long et al., 2003). Spaw is exclusively expressed on the left side of the embryo at some distance from the cardiac cone (Fig. 2A). Within the left cardiac field, Spaw activates several target genes, most likely due to long-range diffusion. That Spaw does not activate target gene expression within the right cardiac field has been attributed to the expression of the secreted Nodal antagonist Lefty1 at the embryonic midline; it acts as an efficient barrier against the diffusion of Spaw (Lenhart et al., 2011; Smith et al., 2011). The other important morphogen cascade, the Bmp signaling pathway (Fig. 2B), has a strikingly re-

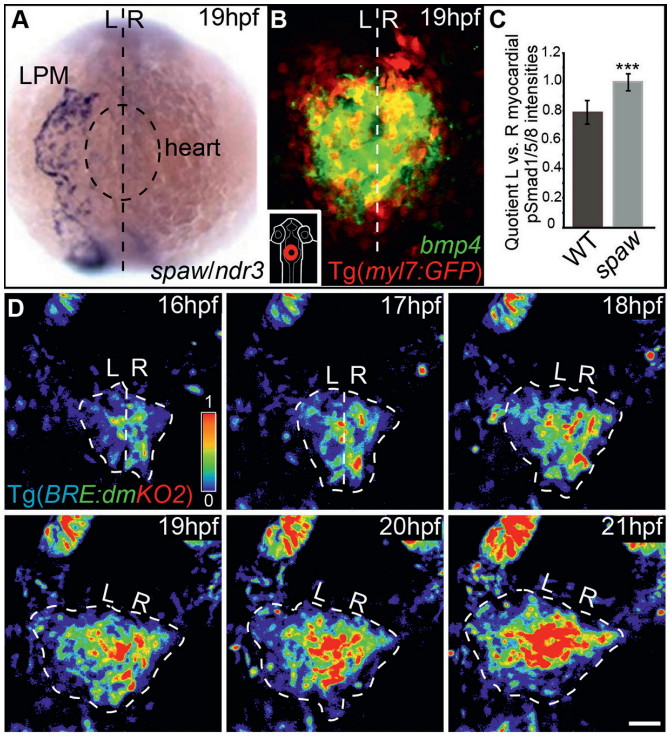

Figure 2:
The L/R asymmetry of Bmp signaling activity depends on Nodal. (A) Whole-mount in situ hybridization shows that expression of *spaw/ndr3* is restricted to the left lateral plate mesoderm (LPM) neighboring the heart cone. (B) In comparison, *bmp4* (labeled green) is expressed throughout the entire heart cone (false-colored in red) as shown by fluorescence two-color in situ hybridization. (C) The L/R asymmetry of pSmad-1/5/8 intensities is abolished upon loss of Spaw/Ndr3. (D) Despite symmetrical *bmp4* gene expression, Bmp signaling activity is higher on the right side of the cardiac field (outlined by white dotted line), as indicated by the Bmp reporter line Tg(*BRE-AAVmlp:dmKO2*)^{mw40} (Veerkamp et al., 2013)

verse asymmetry of activity (Fig. 2C,D). Transgenic Bmp response element (BRE) reporter fish (Collery and Link, 2011) indicated that the intensity of pSmad-1/5/8 activity (those pSmads are activated by the Bmp signaling cascade) is significantly higher on the right side of the cardiac cone (Veerkamp et al., 2013; Fig. 2D). Hence, both Nodals and Bmps establish complementary asymmetries of activity within the cardiac field.

Such a complementary pattern could be due to an antagonism between the two signaling cascades. Three lines of evidence support the hypothesis that left-sided Spaw has an inhibitory effect on Bmp activity: First, the loss of Spaw abolishes L/R differences in Bmp signaling activity. Second, the misexpression of Spaw in single myocardial cells suppresses Bmp signaling activity. Finally, in converse experiments, the loss of Bmp activity in myocardial single cells does not influence the expression of Nodal target genes within the heart field. These and other functional tests suggested that Nodal signaling negatively affects the activity of Bmp within the cardiac field, raising the question of the mechanism by which it does so.

In principle, any mechanism by which Spaw biases Bmp signaling activity should also involve Spaw targets that affect cardiac laterality. Candidate gene approaches revealed that the negative modulation of Bmp signaling activity by Spaw is mediated by the Nodal target Hyaluronan synthase 2 (Has2), an extracellular matrix (ECM)-modifying enzyme which is asymmetrically expressed within the cardiac cone and which is required for cardiac laterality (Smith et al., 2008; Veerkamp et al., 2013). The enzyme Has2 is required for the production of hyaluronic acid, an important ECM component that becomes cross-linked with various proteoglycans. Clonal misexpression of Has2 within single cells of the cardiac field results in a significant local dampening of Bmp signaling activity. Hence, Has2 has a local effect on Bmp signaling activity, implying that the local hyaluronic acid-proteoglycan composition of the ECM is inhibitory for autocrine Bmp signaling. In part, such an effect could be due to a scavenger function of these ECM components for bioactive Bmp ligands on the left side of the cardiac field.

Bmp promotes epithelial and antimotogenic states among cardiac progenitor cells

During zebrafish cardiac development, the behavior of cardiac progenitor cells appears to be tightly controlled by Bmp activity and to shift from a non-motile epithelial state to motile mesenchymal-like states. Cell shape analyses combined with quantifications of cardiac progenitor cell motility rates revealed that high levels of Bmp activity correlate with more epithelial, less motile properties. Comparative microarray expression analyses using highly enriched cardiac tissue helped to identify the Bmp target genes involved in regulating these cellular properties. Many genes that are positively regulated by Bmp encode cell adhesion factors or determinants of epithelial character. A particularly intriguing target gene of Bmp is encoding nonmuscle myosin II (NMII), important in epithelial remodeling, cellular motility, and cell polarity (Conti and Adelstein, 2008; Widmann and Dahmann, 2009; Lecuit et al., 2011). Consistent with positive regulation by Bmp, higher levels of phosphorylated NMII (the activated form of this motor molecule) are present on the right side of the cardiac cone. Both loss- and gain-of-function approaches for NMII activity revealed that NMII is indeed an important regulator of cardiac laterality. Thus, cardiac L/R asymmetry can partly be explained by an antimotogenic Bmp activity that controls levels of NMII, thereby affecting both cell shape and cell motility. In turn, Bmp activity is modulated by asymmetrically expressed Nodal, which conditions the ECM composition within the left cardiac field (Veerkamp et al., 2013; Fig. 3).

Generating invariant organ form involves random cell motility gradients

Remarkably, when single cells misexpress Spaw, dominant-negative Bmp receptor, or constitutively-active Myosin light chain

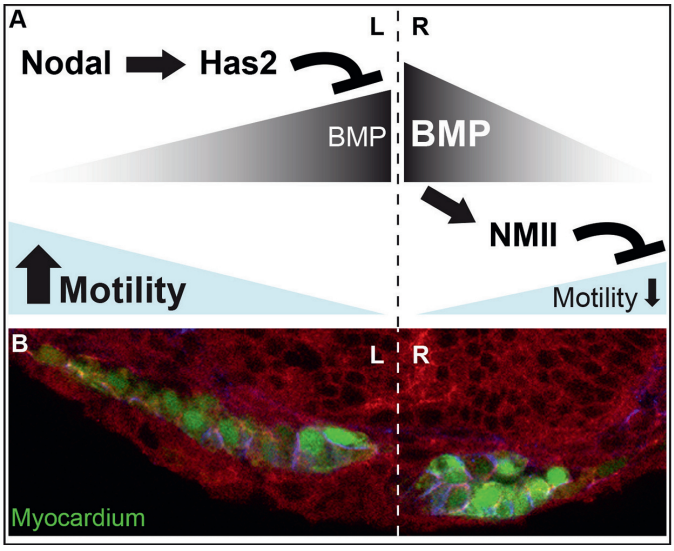

Figure 3:
Cardiac laterality depends on Nodals and Bmps. (A) Schematic diagram illustrating that the Nodal target Hyaluronan synthase 2 (Has2) dampens Bmp activity within the left cardiac field. Reduction of Bmp signaling on the left causes lower expression of non-muscle myosin II (NMII) and higher cardiac progenitor cell motility, which causes leftward directed asymmetric organ displacement. (B) Cross section through the cardiac cone in a myocardial reporter Tg(*myl7:EGFP*)^{twu34} transgenic embryo (myocardial cells marked green; F-actin, red) shows L/R differences of myocardial morphology (Veerkamp et al., 2013).

kinase (caMLCK) - one of the regulatory proteins that phosphorylate and activate NMII - the laterality of the entire cardiac field is affected. In the most extreme cases, such misexpression clones even cause an inversion of cardiac laterality, or situs inversus. That such inversion phenotypes can occur even in the presence of normal left-sided Nodal signaling has several important implications for our understanding of cardiac laterality. This finding implies that complex organ morphogenesis can be explained as the net sum of individual cell behaviors within tightly coherent epithelial groups of cells. It also suggests that the laterality of the entire organ is not strictly predetermined, which would argue against the existence of left-sided guidance cues for cardiac progenitor cells. Could the highly stereotypical morphogenesis of cardiac form instead be explained by a random motility gradient that drives laterality? Random motility gradient models have been used to describe the process of chicken axis elongation (Bénanzéraf et al., 2010).

Mathematical modeling suggests that complex organ form and cardiac laterality can be explained by slight differences in the biomechanical properties of individual cells, as long as these cells are coherently organized. Since the cardiac cone has an epithelial character, motility differences of single progenitor cells can influence the entire group of cells and hence organ laterality. We performed simulations of this process based on the assumption that cells on both sides of the embryonic midline can freely and randomly move in any L/R direction, with cells on the left side moving slightly faster. Invariantly, simulations using these parameters resulted in a robust leftward displacement of the coherent cardiac epithelium (Veerkamp et al. 2013). This model explains an apparent paradox: right-sided cardiac progenitor cells that are not directly affected by Nodal still respond with leftward motility. In principle, the faster rates of motility among cardiac progenitor cells on the left can pull the entire cardiac tissue in their direction. Thus individual, random cell motility is the decisive force during the establishment of cardiac laterality and can be more decisive than left-sided Nodal signaling.

Conclusion

Our work outlines a novel mechanism by which Nodals and Bmps regulate cardiac L/R asymmetry in zebrafish. The principal mechanism involved in this process is an antimotogenic Bmp activity, which is negatively affected by Nodal. It comes as a great surprise that such a well-choreographed and invariant organ morphogenetic process is indeed based on a constant tug-of-war between individual cardiac progenitor cells. This suggests that some of the other wonderful structures that arise from morphogenesis will also turn out to be the result of individual, random cell behaviors rather than a predesigned blueprint.

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I would like to thank all former and current members of my lab and Russ Hodge for their contributions to our research and for continuous discussions of this topic. In particular, I would like to thank Stefan Rohr and Justus Veerkamp who were the driving forces behind the work presented in this review. I am currently supported by a Heisenberg Fellowship of the DFG.

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Salim Abdelilah-Seyfried studied Biology and Biochemistry at the Heinrich-Heine University Düsseldorf, the Manchester Institute of Science and Technology, and the Eberhard Karls University Tübingen from 1988-2003. For his Diploma and Ph.D. thesis work, he joined the lab of Wolfgang Driever at Harvard University (USA) where he participated in large-scale zebrafish developmental screens. After obtaining his Ph.D. in 1996, he started his postdoctoral work in the lab of Yuh-Nung Jan at the University of California in San Francisco where he worked on Drosophila and zebrafish cellular polarity. Since 2002 he has been heading his own research group "Zebrafish Cardiovascular Developmental Genetics" at the Max Delbrück Center for Molecular Medicine in Berlin-Buch. In 2010, he was awarded a Heisenberg Fellowship of the DFG.

Repetitive sequences – an obstacle for the translation

Paul Saffert, Zoya Ignatova

Each codon is translated with different speed

Translation is the last step in converting the genetic information into specific physiological function. The major player in this process is the ribosome, which, with the help of various factors (including initiation, elongation and termination factors), translates the information stored in mRNA codon by codon into a polypeptide. The degeneracy of the genetic code allows for more than one possibility to encode for one amino acid: 61 sense codons are used to encode for 20 amino acids. Synonymous codons (i.e., codons coding the same amino acid) are not uniformly used: some codons are more frequently used than others (1). To each single codon a cognate transfer-tRNA (tRNA) pairs which is loaded with an amino acid by the corresponding aminoacyl-tRNA-synthestase (2). The tRNA species largely differ in their concentrations (3-4). Since charged tRNAs arrive at the ribosome solely by diffusion, the concentration of each tRNA species will significantly influence the rate of translation of each codon (5); codons read by more abundant tRNAs will be translated faster as opposed to codons paring to minor tRNAs. In unicellular organisms the relative abundance of codons correlates well with the tRNA gene copy number (3-4). In multicellular organisms, however, the tRNA concentration differ significantly between various tissues (6), despite the uniform codon usage and common tRNA gene set of each cell. Codon distribution along the mRNA determines the speed of translation of each mRNA; clustering of codons read by lowly-abundant tRNAs causes transient pauses in the ribosome movement (7). This non-uniform distribution of slow and fast translated codons determines the speed of translation initiation (8), guides co-translational folding of the nascent protein (9-10), translocation or membrane insertion (11).

Translation is highly dynamic process and high demand of a certain codon or codon group may reduce the apparent concentration of the cognate charged tRNA, thus artificially converting an abundant codon in an apparent rare codon (5, 12). This depends on the initial concentration of a tRNA species in a cell or tissue. The effect is expected to be non-uniform for each tRNA and might be restricted to only certain tissues of a multicellular organism.

CAG repeat diseases

Huntington's disease (HD) is an inherited neurodegenerative disorder, which is associated with an expansion of a CAG stretch, encoding for glutamine, within the N-terminal exon 1 of hun-

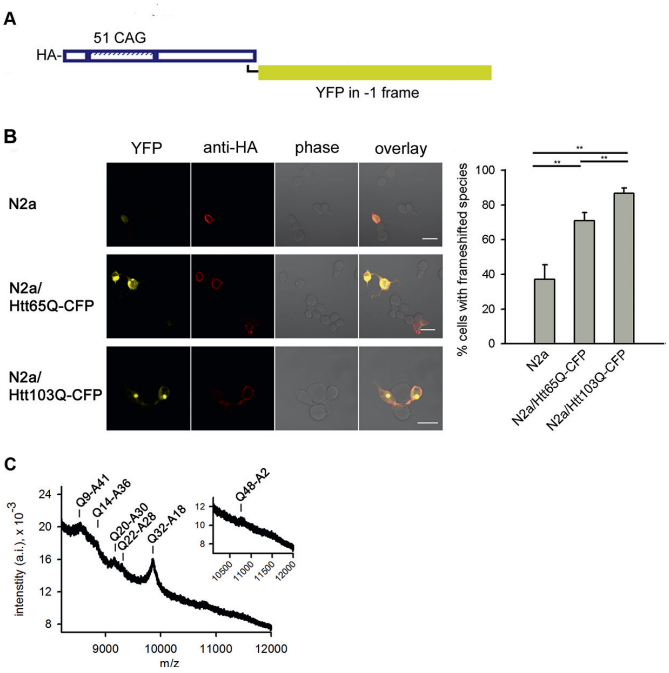

Figure 1. CAG repeats are prone to translational frameshift at any position within the CAG stretch:
(a) Schematic of the reporter of -1 frameshifting, Htt51Q(-1)YFP.

(b) N2a, N2a/Htt65Q-CFP, and N2a/Htt103Q-CFP cells were transiently transfected with the Htt51Q(-1)YFP reporter and visualized by fluorescence (YFP) and phase contrast (phase) microscopy after 24 hr (left panel). Htt51Q and Htt51QYFP expression was monitored by immunostaining of the N-terminal HA tag. Scale bar, 10 mm. The percentage of cells containing YFP-positive, frameshifted aggregates from the total amount of cells transfected with Htt51Q(-1)YFP (i.e., HA-positive) was quantified from the microscopy images (right panel). The values are expressed as means of five independent experiments ± SEM. **p < 0.01.

(c) MALDI-TOF-MS analysis of N2a/Htt103Q-CFP cells expressing Htt51(-1) YFP for 24 hr, coimmunoprecipitated with GFP-antibodies, subsequently separated by 2D-gel electrophoresis. Figure adopted from (19).

tingtin (Htt) protein (13). Mutation-based expansion of the CAG stretch over a threshold of 37 consecutive codons increases the propensity of Htt to aggregate in a CAG-length-dependent manner (14). HD is associated with selective neuronal loss with the highest vulnerability of the striatal neurons even though Htt is ubiquitously expressed in the whole organism (15). The mechanism of specific targeting of the striatial neurons remains enigmatic. At later pathology stages, insoluble aggregates in nucleus or cytoplasm of the disease-damaged tissue are built (16). Whether the aggregation per se triggers pathology or rather small soluble pre-aggregates cause cellular disfunction, is still a matter of intense debate. Interestingly, along with the polyQ aggregates in neuronal tissues, polyserine (polyS) and polyalanine (polyA) species have been detected within the damaged neurons of diseased individuals (17). The polyQ stretches are exclusively encoded by the CAG codon even though another codon (CAA) also codes for glutamine.

Translation of repetitive stretches may cause abnormal translation activities, including translation frameshifting. Translational frameshifting is a recoding event in which the ribosome is forced to move to one of the alternative reading frames and continuous to translate this frame instead of the original 0 frame (18). Translational frameshift within the CAG repeat in +1 and -1 direction would result in AGC- and GCA-encoded stretches encoding for serine and alanine, respectively. Whether these polyS and polyA species detected post mortem in the patients resulted from a translational frameshift is still unknown. Thus, we sought to investigate the frameshifting propensity of repetitive CAG stretches within Htt exon 1. Indeed, expanded CAG stretches are highly prone to frameshifting and the shift to -1 reading frame (i.e., encoding polyAla) is more frequent (19). Performing experiments to mechanistically understand the frameshifting within expanded CAG stretches we came to a rather surprising observation: the depletion of the cognate, charged glutaminyl-tRNAGln-CUG is the main cause for -1 frameshifting within expanded CAG repeats.

The amount of CAG codons determines the frameshifting frequency

To investigate the frequency of frameshifting, we used a reporter system, in which the YFP gene is fused in -1 frame to Htt exon 1 with 51 CAG repeats (Figure 1A); -1 frameshifting will lead to YFP expression. CAG-repeat expansion increases the susceptibility of Htt to intracellular proteases, releasing exon 1 comprising the CAG repeat (20) which has much higher propensity to aggregate and dominates the aggregates in the disease-damaged tissues of patients (21). Thus, in our experiments we used only exon 1 with various CAG lengths. The reporter construct, Htt51Q(-1)YFP, was ectopically expressed in murine neuroblastoma cell line (N2a) stably expressing different CFP-tagged Htt constructs, Htt65QCFP or Htt103QCFP (22). In all cells we detected YFP-positive species whose number was the highest in cells expressing

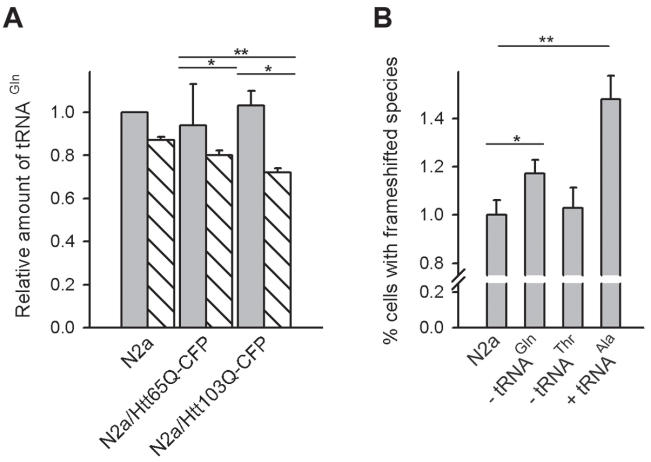

Figure 2. Concentration of charged, glutaminyl-tRNAGln-CUG decreases in a CAG-length dependent manner:
(a) Total (gray bars) and aminoacylated-tRNAGln-CUG (dashed bars) levels quantified from the Northern blots of various N2a cells expression Htt51Q(-1)YFP reporter. The intensity of total tRNAGln-CUG of each sample is normalized to the intensity of tRNAGln-CUG of the control N2a cells. Glutaminyl-tRNAGln-CUG is determined as a fraction of the total tRNAGln-CUG in each sample. Values are mean ± SD of 3 independent experiments. * for $p < 0.05$, ** for $p < 0.01$.

(b) tRNAGln-CUG (- tRNAGln) and tRNAThr-AGU (-tRNAThr) were partially silenced (appr. 40%) with siRNAs. tRNAAla-UGC was upregulated by transfection with in vitro transcribed tRNAAla-UGC (+tRNAAla). The frameshifting is represented as the percentage of cells (± SEM) containing YFP-positive aggregates in the total population of cells transfected with Htt51Q(-1)YFP (i.e., HA-positive) and compared to the control cells (N2a) for which the percentage of frameshifted cells was set as 1 (as in Figure 1B). * for $p < 0.05$, ** for $p < 0.01$. Figure adopted from (19).

Htt103QYFP protein (Figure 1B). To our surprise, YFP-positive spots reporting on frameshifted species appeared in the wild-type N2a cells transfected only with the Htt51Q(-1)-YFP reporter (Figure 1B). Importantly, the increased translation of CAG codons in cells expressing Htt variants with longer CAG stretches correlated with the frequency of frameshifting. The YFP-positive species resulted from a frameshifting within the CAG stretch as determined by mass spectrometry (Figure 1C).

Hybrid polyQ/polyA species with different Q:A ratio were formed (Figure 1C), suggesting that -1 frameshifting occurred stochastically at any codon within the CAG repeat. This raised the intriguing question as to whether the glutaminyl-tRNAGln-CUG is depleted while translating long consecutive repeats. Measurements of tRNAGln-CUG revealed no changes in the total concentration of tRNAGln-CUG, however, a significant decrease of the level of charged glutaminyl-tRNAGln-CUG (Figure 2A), implying that an increased, simultaneous translation of CAG codons in the cell reduces the concentration of translationally competent aminoacylated tRNA. Furthermore, we decreased the tRNAGln-CUG using the siRNA approach. Decrease of the tRNAGln-CUG enhanced frameshifting (Figure 2B). This effect is specific, as al-

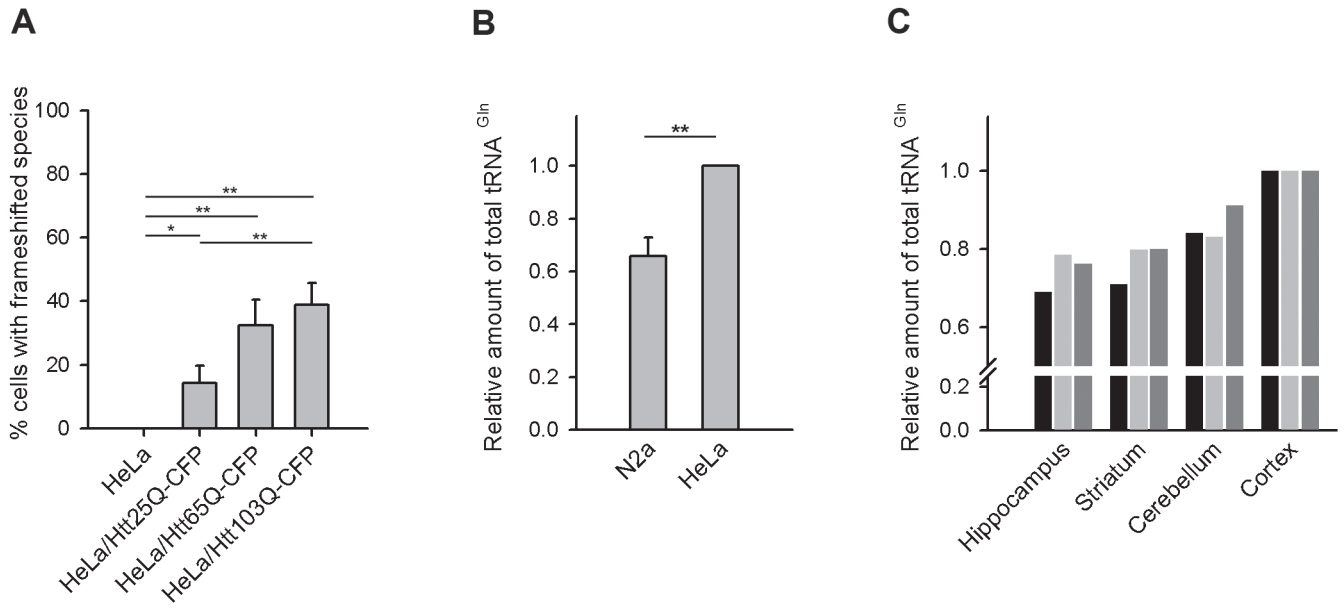

Figure 3. tRNAGln-CUG differs in different cells and tissues:
(a) Quantification of frameshifting frequency within the CAG repeat in various HeLa cell lines ectopically expressing Htt51Q(-1)YFP reporter and visualized after 24 h by fluorescence microscopy (as in Figure 1B). Values are expressed as means of > 5 independent experiments ± SEM. * for $p < 0.05$ and ** for $p < 0.01$.
(b) Northern blot analysis of the total tRNAGln-CUG isolated from N2a and HeLa cells. The intensity of the tRNAGln-CUG band is related to the intensity of 5S rRNA and the values are shown as a mean ± SD of 3 independent experiments. The values of HeLa cells were arbitrarily set as 1. ** for $p < 0.01$.
(c) Total tRNA concentration in different brain tissues of three mice (differently colored bars for each mice) measured by northern blot. Figure adopted from (19).

terations of tRNA which is unrelated to the translation of CAG stretches, tRNAThr-AGU, had no effect (Figure 2B). Interestingly, an increase of tRNAAla-UGC, which pair to the GCA codon when the ribosome shift the CAG-reading frame to -1 frame, significantly increased the frequency of frameshifting (Figure 2B) suggesting a competition between the glutaminyl-tRNAGln-CUG and alaninyl-tRNAAla-UGC for the A-site of the translating ribosome as a mechanism for translational frameshift.

tRNAGln-CUG concentration differs in various cells and tissues

Clearly, the simultaneous translation of many CAG codons decreases the effective concentration of the cognate tRNA leading to aberrancies in translation. The tRNA sets vary from cell to cell (6), which raised the question as to whether frameshifting frequency differs in different cells or tissues. To investigate this we transfected the Htt51Q(-1)YFP reporter in different HeLa cell lines that also stably express CFP-tagged variants of Htt, Htt25Q-CFP, Htt65Q-CFP and Htt103Q-CFP (22). We observed YFP-positive species reporting on the -1 frameshifting (Figure 3A); however the amount of them was much lower compared to the corresponding N2a cell lines (Figure 1B). Notably, unlike in the N2a cells, we did not observe any frameshifting in HeLa cells expression only the Htt51Q(-1)YFP reporter (Figure 3A). Comparison of the tRNAGln-CUG amount in both cells showed much higher concentration of tRNAGln-CUG in HeLa than in N2a cells (Figure 3B). Thus the higher frameshifting frequency in N2a cells cells mirrors the difference in the tRNAGln-CUG concentration between two cell lines, implying that the tRNAGln-CUG concentration is the main cause for frameshifting when translating extensive amounts of CAG codons. Given the higher vulnerability of selective loss of the striatal neurons in HD pathology than of other neuronal tissues, we next asked whether the concentration of tRNAGln-CUG differs in striatum. Comparison of tRNAGln-CUG in four neuronal tissues revealed one of the lowest concentrations in striatum (Figure 3C). The frequency of the frameshifting in these neuronal tissues is currently under investigation.

Translational frameshifting: implications for the HD pathology

Taken together, our results suggest that simultaneous translation of large amount of CAG codons leads to aberrancies in translation (Figure 4). Increased demand for glutaminyl-tRNAGln-CUG creates a bottleneck which results in -1 frameshifting within the CAG stretch. Every codon in the CAG repeat is equally susceptible to frameshifting leading to a formation of a cohort of transframe encoded species with hybrid polyQ/polyA stretches which differently modulate the conformational switch to nucleate fibrillization of the parental polyQ protein (19). This effect strongly depends on the Q:A ratio generated in each CAG repeat stretch upon framehsifting (19).
A direct experimental determination of the frameshifting fre-

quency of endogenous Htt is neurons is still missing, but presence of polyA or polyS proteins in HD-affected brain tissues (17) and the intrinsic high propensity of frameshifting in neurons (23) support the notion, that frameshifting may accompany HD pathology. The striatum is an early target of HD, and striatum has one of the lowest tRNAGln-CUG concentrations among the brain regions of mouse. Furthermore, the CAG repeats have the highest instability in striatum; the CAG stretches are by approximately 10 codons larger than the CAG repeats in any other tissue of the same individual (24). Thus, is it conceivable to think that the combination of expanded CAG stretches and the lower tRNAGln-CUG household would potentiate the frequency of frameshifting of expanded CAG repeats in striatum. Thus, frameshifting within expanded CAG stretches may act as modifier of HD pathology and may contribute to the heterogeneity in disease course and onset on both cellular level and single individual.

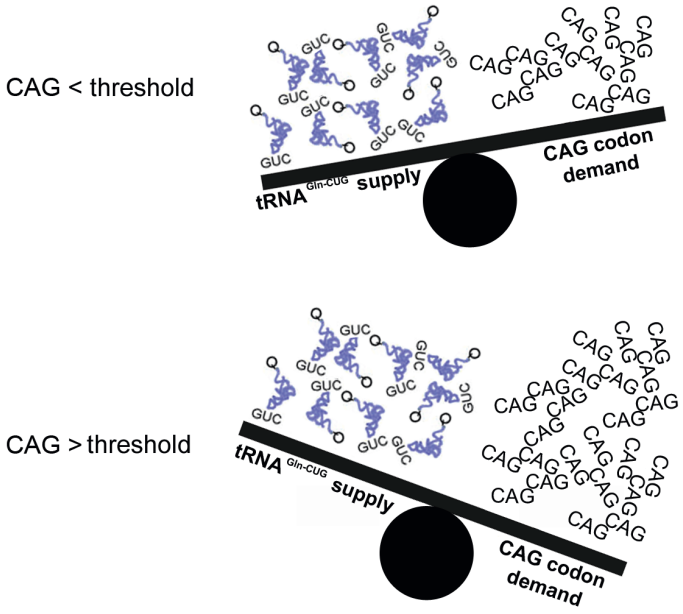

Figure 4. Disbalance between the demand and supply for glutaminyl-tRNAGln-CUG alters translation of repetitive CAG stretche: Abnormal expansion of CAG repeats, over the HD disease threshold, increases disproportionally the demand of glutaminyl-tRNAGln-CUG which causes translational frameshift within the CAG stretch.

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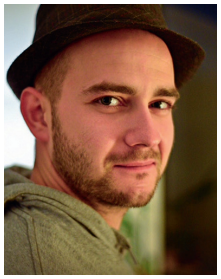

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The 4th "Physics of Cancer" Symposium

Leipzig, September 24 – 27, 2013

Like in the three meetings before, this year's meeting again intends to bring together researchers from the worldwide pioneering groups that are concerned with investigating the physical mechanisms underlying cancer progression. As a special feature, the organizers have joined forces with the DGZ and announce the symposium as Special Interest Meeting of the DGZ.

Aim of the Meeting

The analysis of physical properties of cells undergoing malignant transformation is a highly important and an emerging field in current cancer research, cellular biophysics, and cell biology. Recent findings in this novel research field revealed that biomechanical properties of cancer cells promote tumor growth, cell motility and metastasis formation within the human body. In the focus of the studies are certain observations regarding biomechanical properties: First, the actin cortex of cancer cells is pronouncedly softer and hence supports elevated tumor growth and enhanced cell division. Second, although the actin cortex softens, the cancer cells can still resist high pressures exerted from the microenvironment, which enables the primary tumor to break through the tumor boundaries and invade into the surrounding connective tissue extracellular matrix. In return, components of the cytoskeleton are pronounced which results in an overall stiffening of the primary tumor. Third, the ability to transmit and generate contractile forces of cancer cells increases their aggressive potential to invade into the connective tissue microenvironment and promote tumor progression and metastasis formation.

Finally, these novel insights have an impact on the understanding of how and why certain cancer cells get the ability to invade into the human body and form metastases at targeted sites. Thus, we are convinced that this 4th Physics of Cancer Symposium will provide state-of-the-art research technologies, high-class knowledge and fruitful discussions.

Topics Include:
- Biomechanics (Biopolymers, Networks, Rheology, Cytoskeleton, Cell Shape)
- Forces, Motion, Adhesion (Cell Motility, Assembly, Molecular Motors, Cell Division)
- Oncology
- Imaging

Claudia T. Mierke, University of Leipzig, Germany
Josef A. Käs, University of Leipzig, Germany
Harald Herrmann, German Cancer Research Center (DKFZ), Heidelberg, Germany
Valerie Weaver, University of California, San Francisco, USA

Registration fee: 200 € – for DGZ members: 100 €.
The number of participants is limited. Please, register early.
All participants are invited to apply for a short talk or a poster contribution by submitting an abstract.

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The paths of migrating cells

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In order to look at dynamic events in life e.g. directed or undirected movements of objects, such as the orbit of a satellite, the diffusion of molecules, or the path of foraging animals, one needs to visualize a development in time and space in one frame. This is done by recording the coordinates of the object and superimposing them on the same background. The connection of those points is called a trajectory and is often used to retrieve information about moving objects. A well known everyday example would be a map of bus stops. We do not only see on one page where the bus is going to stop, but we can also use this bus-trajectory to calculate the bus speed, to see if it's reaching a certain place in the most straightforward way and we can even use this information to predict the position of the bus at a precise time. By analyzing trajectories we do not only get information about the instantaneous or mean speed of an object, but also about the directionality of the path, for example how much the object is turning during a certain time-window or for how long it is moving in the same direction. This information is described by the persistence of an object and defines if the object is moving in a directed motion or rather in a random path. Even a dynamic evolution within the trajectory – for example an object moving at different speeds in different stages – can be identified, theoretically described, predicted and probably being interfered with. Such analysis are not only used for buses but are found in all areas of our daily life, such as tracking population, money or recently you can even download apps for your smartphone to see the phone trajectory when it's getting stolen!

Likewise, the position of living cells can be recorded during cell migration and cell trajectories can be drawn. This in generally is done to investigate fundamental migration properties and to compare the migration behavior of different cell types, for example in wound healing essays (Payne, Bhalla et al. 2011) or in the study of cancer.

The first reports about trajectories of cells are from the 1970s, where researchers started to track axons in the brain (Stirling 1978), or analyzed the trajectories of the slime-mold amoebae and granulocytes with and without chemo tactical cues (Mato, Losada et al. 1975; Hall 1977; Hall and Peterson 1979). Cell trajectories are generally the way to describe the migration of cells, allowing to compute their speed or their persistence. There are several types of migration, cathegorized by cells which do need adhesion for migration (mesenchymal migration) or cells which move with no or very low adhesion. This migration type was first found in amoebae and was therefore called amoeboid migration (Friedl and Weigelin 2008; Lämmermann and Sixt 2009; Guck, Lautenschlager et al. 2010). Those two migration modes are very different and can be – besides other factors – characterized by their cellular trajectories. However, cells do not necessarily belong to one or the other group of migration and can – by changing their chemical or physical environment – switch between those two migration modes (Bergert, Chandradoss et al. 2012). The change of the physical environment can actually bring cells, which would not move otherwise, become very motile. This is the case for dendritic cells of the immune system, which only start to migrate when they are in a confined environment, without the need of integrin adhesion (Faure-Andre, Vargas et al. 2008; Friedl and Weigelin 2008; Lammermann, Bader et al. 2008; Heuze, Collin et al. 2011). Dendritic cells in confinement move in an amoeboid manner and have already been studied in different environments such as gels or one dimensional channels, or tissues (Faure-Andre, Vargas et al. 2008; Lammermann, Bader et al. 2008). However, the migration of cells in collagen gels is very hard to control and difficult to image and cells in channels only offer one dimensional data. Therefore, we developed a new system in order to confine cells in a two dimensional way. In our arrangement we can very precisely define the height of a roof on top of the migrating cells, which we generally tune to be between 3 and 10 um. We also modified our setup in order to have large fields of view of migrating cells which we can observe over a long period of time (up to 48 hours) and study long trajectories of these cells. In addition to questions generally asked in migration assays such as the speed and persistence of cells, we are interested in a very specific information of trajectories: the search behavior of dendritic cells. Why do we expect dendritic cells to search for something? The main task of these cells migrating throughout the body is to uptake possible threats, such as viruses, bacteria

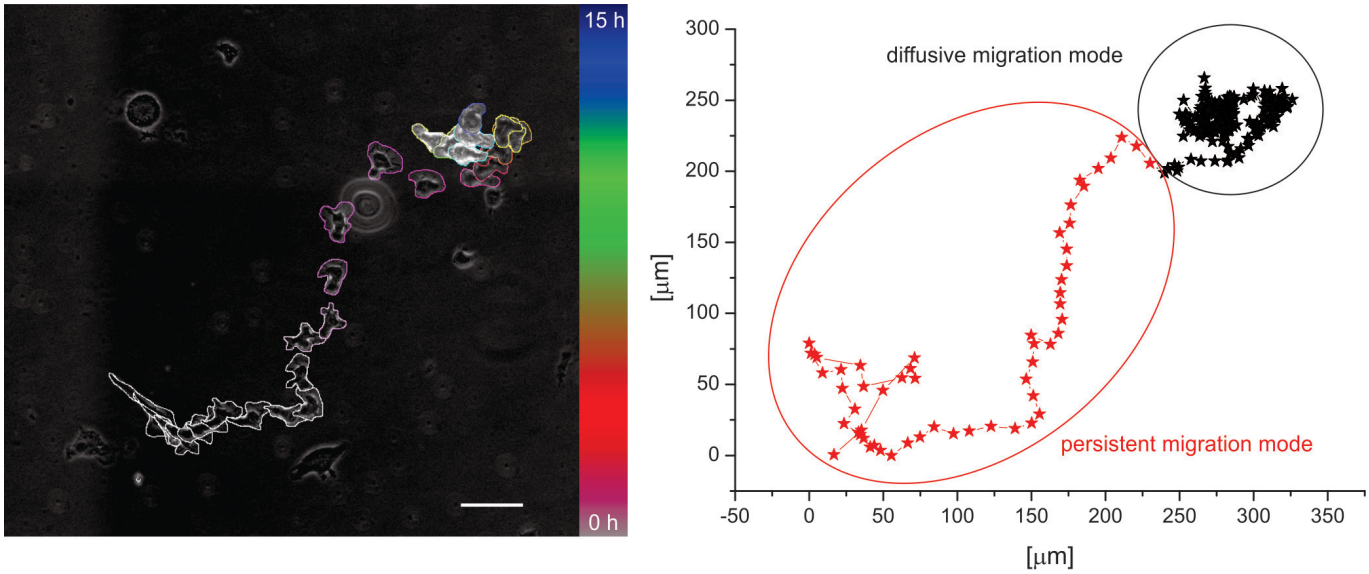

Figure 3:
Example of intermittent migration trajectory over a time course of 15h. Left: Overlay of phase contrast images of migrating dendritic cell. Scale bar 50 um. Right: Trajectory of the same dendritic cell where persistent (red) and diffusive (black) migration modes have been identified.

or other pathogens and to alert the immune system. However, to take something up, it needs to be found first. Here finding means two things: the physical encounter, and then the uptake by the dendritic cell. So do dendritic cells really search for pathogens or does this happen in a rather random manner? We are looking into the trajectories of dendritic cells in order to understand their behavior and to compare it to theoretical models. Different models exist which all describe search behavior. However, they differ in their parameters and therefore settings of the system. For example, if a searcher is trying to find random objects which do disappear forever once he found them, then he might just have the most success by walking straight and picking them up on his way. However, such a strategy might not be the most successful if the object can reappear after it was found once, in which case so called levy walks might be better (Viswanathan, Buldyrev et al. 1999; Bartumeus, Catalan et al. 2002; James, Plank et al. 2008). But if targets are not randomly but instead regularly distributed, another theory describes an optimized search strategy, which is a simple persistent random walk (Tejedor, Voituriez et al. 2012). However, for some searchers there is a specific difficulty: they cannot move and 'find' at the same time. In daily life this might be best illustrated by a person having lost its key on a sandy beach: in order to cover some area the person needs to move, but since the key might be well hidden in the sand, he also needs to stop moving, kneel down and actually look for the key. Such biphasic search behavior is described in intermittent search strategies (Benichou, Loverdo et al. 2006; Benichou, Loverdo et al. 2011). The fast moving phases of the searcher in this theory are described as persistent, the so called ballistic phases and the time while the searcher is staying in one area to take up objects by a random, the so called diffusive movement. We use single cell trajectories to investigate which way dendritic cells are migrating in order to sample their environment best. Since dendritic cells have been shown in 1D microchannels to show a biphasic migration behavior (Faure-Andre, Vargas et al. 2008), they might indeed use an intermittent search strategy in order to look for pathogens. To investigate this question, we analyze our 2D cell trajectories not only in a conventional way, but we also look out for different modes of migration within one cellular migration track (fast and persistent parts versus slower and diffusive parts). An example of such an intermittent migration track is given in Figure 1. The distinction between different modes is done by analysis of the means square displacement of the trajectories. A comparison of our data with theoretical models will enable us to find out if cells are searching and if they do, which search strategy they are using? If cells do indeed have a specific strategy it will be interesting to see if this is adapted and optimized for certain types of environments.

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External and internal forces regulate the transendothelial migration and invasion of cancer cells from solid tumors

Claudia Tanja Mierke

Introduction

The motility of cancer cells from solid tumors into the extracellular matrix of connective tissue has long been investigated by genetic expression profiling and mutational analysis. This screening has been focused on the analysis of genetic alterations affecting cell-matrix and cell-cell adhesion molecules, focal adhesion proteins, cytoskeletal proteins and their signaling pathways during the progression of cancer disease and has revealed numerous of candidate genes playing a role in cancer. In particular these candidate genes have been obtained by comparing gene expression profiles of invasive and non-invasive cancer cells. Indeed, many proteins have been identified to play a role in cancer cell invasion, transendothelial cell migration and hence tumorigenicity depending on the specific cancer type. However, a clear, unique picture of cancer progression is still elusive. At this point, biophysics has been introduced into cancer research and revealed that biomechanical properties of cancer cells may play a role in cancer progression and in particular in cancer cell invasion and transendothelial migration (1-6). The biomechanical properties of the external microenvironment of neoplasms and tumors as well as the internal mechanical properties of the cancer cells can affect and regulate the invasiveness of cancer cells into the extracellular matrix microenvironment and the transendothelial migration into blood or lymph vessels (Figure 1). In more detail, it has been suggested that biomechanical properties of cancer cells such as the transmission and generation of contractile forces is determined by the altered mechanical properties of the tumor microenvironment compared to "healthy" normal tissue. Hence, the analysis of the biomechanical properties of the local tumor microenvironment such as the extracellular matrix and embedded neighboring non-cancer cells such as endothelial cells has become a main focus of biophysically based cancer research.

Besides external forces, internal forces of cancer cells regulate the invasiveness and the transendothelial migration of cancer cells. These internal forces may result from cytoplasmic restructuring of the cell`s cytoskeleton for example by breaking covalent-bonds or van der Waals interactions, forming new actin or keratin polymers or establishing new connections. For example, the focal adhesion protein vinculin has been shown to be involved in the regulation of contractile forces as well as the cell surface receptors such as the integrin $\alpha 5\beta 1$ and the glycosyl phosphatidylinositol anchored transmembrane protein CD24 regulate the transmission of contractile forces (7-10). Many cellular functions such as cell cycle progression, cell survival, and migration of cells have been associated with biomechanical properties demonstrating the importance of biomechanics in the field of cell biology, molecular biology and biochemistry. Thus, this article discusses how the external forces applied to both normal and cancer cells as well as internal forces of cancer cells determine their invasiveness into extracellular matrices or their transendothelial migration efficiency. Finally, this article highlights by addressing the impact of the regulation of external microenvironmental forces generated by the extracellular matrix or external forces exerted by neighboring endothelial cells on cancer cells, which then change the cancer cell's cellular biomechanics, and subsequently the cancer cell's motility or invasiveness and cancer cell's ability to cross endothelial borders.

External forces regulate the behavior of cancer cells

The invasiveness of cancer cells depends not only on a single biomechanical or biochemical parameter, it rather depends on the balance of several parameters that act together to facilitate cell invasion and transendothelial migration. In particular, the stiffness or softness of cancer cells influences together with the cytoskeletal remodeling dynamics and the transmission or generation of contractile forces the invasiveness of cancer cells into 3D extracellular matrices (2, 5, 8).

Forces exerted by the extracellular matrix

The microenvironment of cells in tissues differs in their composition, steric hindrance (pore-size, bending stiffness, fiber

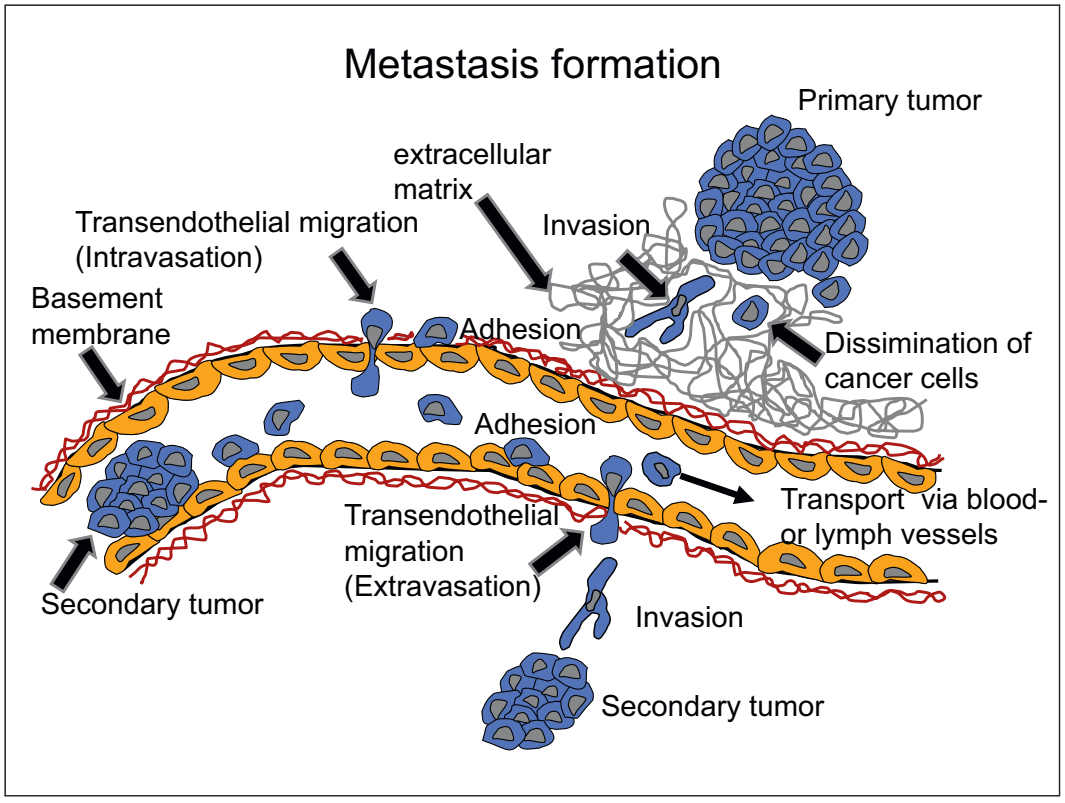

Figure 1: Steps of malignant progression of cancer. Cancer cells spread from the primary tumor and invade into the extracellular matrix of connective tissue. Some cancer cells transmigrate through the endothelial cell layer of blood or lymph vessels including the basement membrane and are transported through the whole body. Then, circulating cancer cells have two possibilities: one possibility is that cancer cells adhere to the endothelium and form a secondary tumor in the lumen of the vessel. The second possibility is that the cancer cells transmigrate through the endothelial cell lining and the basement membrane into the connective tissue of targeted organs and form a secondary tumor (so-called process of metastasis).

thickness) and thus, mechanical properties between the various parts of the body are different. In tumors, the microenvironment is altered compared to "healthy" normal connective tissue: for example the stiffness of the extracellular matrix and resulting tissue deformations are almost modest in cancerous tissue and serve as mechanical marker for primary or secondary tumors. A mechanical parameter of the extracellular matrix is the stiffness, which is a measure of the resistance exhibited by the elastic extracellular matrix upon deformation. The mechanical properties of the extracellular matrix are important in regulating the functional behavior of cancer cells by inducing mechano-sensitive signaling pathways. During the malignant progression of cancer the biomechanical homeostasis of the tissue is often deregulated, and tumors exhibit increased stiffness compared to "healthy" normal tissues (11). The increased stiffness of tumors alters the tensional homeostasis of cancer cells and increases their ability to transmit and generate contractile forces (11). An increase in the stiffness of the extracellular matrix of connective tissue has been implicated in tumorigenesis and thus the interaction of a tumor with its stroma may shed light on the process of malignant tumor progression. In contrast, the optical deformability of cancer cells determined by using an optical stretcher device is increased in metastatic cancer cells compared to "healthy" cells, but consistent to Paszek et al. the cellular contractility is increased in metastatic cancer cells compared to "healthy" normal cells (12). However, the interaction of mechanical and biochemical signals of the matrix and cancer cells and how this is connected to genetic and epigenetic alterations in tumor progression and metastasis is still elusive. Finally, one question remains still open: How may this connection between mechanical, biochemical and genetic processes contribute substantially to the understanding of tumor progression? How will this knowledge renew the current view dramatically and the classical proposed hallmarks (13-14), which definitely exclude the mechanical properties of all components involved: the cancer cells, neighboring cells and the extracellular matrix microenvironment (13)?

Revealing its physical nature the extracellular matrix senses cancer cells for alterations in stiffness by inducing mechano-regulatory pathways. One important molecule in acting as a mechano-sensor is vinculin (8-9). In particular, cells are able to adhere to the extracellular matrix of connective tissue through cell-matrix adhesion molecules. All cell-matrix adhesions contain integrins as their major transmembrane receptors (16-17), which are able to transmit forces (protrusive forces or adhesive forces) derived from the microenvironment to the interior of the cell, and in turn cytoskeleton-generated forces are exerted to the exterior microenvironment. Compared to stationary cells, which are firmly adhered to the extracellular matrix components and where external and internal forces are of the same amount and lead to non-polarized cells, motile cells are characterized by an unequal force distribution, which polarizes the cell by promo-

ting cell contraction, extension and translocation in a specific direction (depending on external signals) (18-19). As a cancer cell moves on or in the extracellular matrix, it experiences external forces, which include the viscous force or resistance from the surrounding matrix and cell-substrate interaction forces, i.e. adhesion forces, and internal forces, i.e. cytoskeletal forces that are generated by the cytoskeleton. As forces are applied through adhesion molecules towards the microenvironment, cells continuously respond by exerting reciprocal contractile forces to external forces, which are applied to cancer cells by the extracellular matrix and surrounding neighboring cells (18). Finally, the anisotropy of the cell's adhesive microenvironment such as the mechanical forces controls the intracellular cytoskeletal organization and facilitates the polarity of the cell (20) and subsequently regulates the invasiveness or transmigration ability of cells in 3D extracellular matrices.

Forces exerted by other neighboring cells within the extracellular matrix

Some years ago the "biochemical" role of endothelial cells in metastasis was established and well defined: the endothelium fulfills the role of a passive barrier avoiding cancer cell invasion and spreading into the blood or lymph vessels (21-22). However, this view changed dramatically in 2008: Since then it is widely accepted that endothelial cells fulfill a novel function during cancer metastasis by facilitating and increasing the invasion of certain "aggressive" cancer cell lines into 3D extracellular matrices (2). Indeed, this finding challenged the view of the endothelium from a passive barrier restricting cancer cell invasion in general to the view of the endothelium acting as an active modulator or enhancer of the invasion of specific cancer cell types (2). However, there are still unsolved steps in metastasis and open questions: Do endothelial exerted mechanical forces regulate the transmigration of cancer cells? Do transmigrating cancer cells sense the mechanical alterations evoked by endothelial cells? What roles play guiding cells such as tumor-associated macrophages in the mechano-regulatory scenario? How do cancer cells that circulate in the blood stream manage to adhere to the endothelium and transmigrate through it? Do cancer cells circulating in vessels use similar mechanisms as leucocytes to transmigrate through the endothelial lining?

However, the transmission and generation of contractile forces by cancer cells are involved in the process of invasion and transendothelial migration. It has been shown that the contractile actomyosin apparatus of adjacent endothelial cells supports the invasiveness of cancer cells (23). Until now it is still elusive whether the intermediate filament cytoskeleton and the microtubule cytoskeleton are involved in transmitting and generating contractile forces to enhance the effect of the actomyosin cytoskeleton. As invasive cancer cells alter the mechanical properties of adjacent endothelial cells, in turn, endothelial cells may alter the mechanical properties of cancer cells to enable them to transmigrate through. How does this interaction take place? Do endothelial cells induce forces to adhering cancer cells? How does the adhesion of invasive and non-invasive cancer cells alter the exerted mechanical forces from the underlying endothelium?

Internal forces regulate cellular functions of cancer cells

Forces can be generated by the actomyosin cytoskeleton of cells and are important in regulating cellular motility through extracellular matrices and the endothelium. By coordinating the entire process of cell locomotion precisely, the cytoskeleton consists of a polymer network including the three main cytoskeletal filaments: actin filaments (microfilaments), intermediate filaments and microtubules. The filaments represent polymers with different mechanical properties such as stiffness or rigidity, which is given by the persistence length of the biopolymers. The persistence length is a basic mechanical property which quantifies the stiffness of a polymer and can be expressed using bending stiffness (the Young's modulus). In particular, the persistence length is defined as the end-to-end distance vector over which the filament can be bent by applying thermal forces. If the stiffness of a polymer increases, the persistence length increases similarly (24).

Forces generated by the actin cytoskeleton

The primary step of starting the motility of cancer cells is the protrusion formation of the leading edge in the direction of the motion. The extension of the leading edge of migrating cells is a multi-step process, which is a precisely regulated complex scenario (25-32). The underlying mechanism of building protrusions is the active polymerization of actin filaments towards the cell membrane at the leading edge that pushes the membrane straight forward in the direction of motion or invasion. However, the polymerizing actin filaments are not able to transmit or generate contractile forces: without any accompanying myosin motors, cells are not able to generate strong forces to mediate the movement of a cell's leading edge. In more detail, an actin filament is not a stiff rod, which stops growing once when reaching the cell membrane. Instead, actin is an elastic filament, which is bent after applying loads or forces, and it may insert itself through the existing actin filament and cell membrane (33). Subsequently, the lengthened actin filament is able to exert an elastic force towards the cell membrane in order to push it forward in the direction of movement.

Indeed, actin filaments and extracellular matrix focal adhesions are needed to omit the backward movement of polymerizing actin filaments. It still remains an open question: How can an actin filament polymerize against an external stress such as the cell membrane or external forces and generate a polymerization force?

The actin filaments are semiflexible polymers, assembled from dimer pairs of globular actin, and functionally polar. Thus, actin filaments have two distinct ends: a fast (plus end) and a slow growing end (minus end). The minus end of the actin filament has a critical actin monomer concentration that is approximately six times higher compared to the plus end actin monomer concentration. Above its critical concentration, the actin filament end will bind actin monomers and grow by polymerization. However, when the concentration of actin monomers is below the critical concentration, actin monomers detach from the actin filament end inducing actin filament depolymerization. Due to these two different critical actin concentrations at the plus and minus ends of the actin filament, these actin filaments are able to grow asymmetrically. In particular, when the actin monomer concentration is in between the two critical actin monomer values, only the plus end of the actin filaments grows while the minus end depolymerizes, a situation termed "treadmilling". This actin treadmilling process explains how actin filaments can generate a polymerization force regulating cell invasion and transendothelial migration.

Forces generated by the intermediate cytoskeleton

Intermediate filaments have a shorter persistence length compared to actin filaments and hence, are much more flexible than actin filaments and microtubules. Although there are different classes of intermediate filaments such as vimentin, desmin, keratins, and lamins, they are in contrast to actin filaments or microtubules not polarized, not able to treadmill and do not normally depolymerize under physiological conditions upon polymerization. Thus, intermediate filaments are more static and less dynamic compared to actin filaments or microtubules. How these intermediate filaments contribute to the mechanical properties is still under investigation. It is suggested that they even play an equal important role similar as actin filaments for providing cellular mechanical properties such as forces.

Forces generated by the microtubule cytoskeleton

Microtubules exhibit the largest persistence length when compared with actin or intermediate filaments and therefore they are the stiffest of these three biopolymers (34). As actin polymers, microtubules are rod-like polymers. The alpha and beta tubulin protein subunits assemble alternating into protofilaments, and typically 13 of these protofilaments align together to form a hollow cylinder providing the strong rigidity or stiffness. Microtubules possess similar assembly and disassembly dynamics compared to actin. In more detail, microtubules are polar regarding their function, undergo readmilling similar to actin filaments and can generate a force upon polymerization of tubulin monomers into protofilaments (35). How microtubules contribute to the motility of (cancer) cells is under strong investigation and may also play a role in macromolecule crowding effects regulating (cancer) cell invasion.

Conclusion

This article focused on the internal forces of cancer cells and on external forces generated by the extracellular matrix connective tissue and/ or adjacent endothelial cells and the impact of internal and external forces on cancer cell invasion and transendothelial migration. Indeed, external and internal forces regulate many functions of cancer cells such as cell proliferation, apoptosis, cell fusion, connective tissue invasion and transendothelial migration. The understanding of the interplay between cancer cells and their microenvironment and in particular, the mechanical stimulation of cancer cells through external forces of the microenvironment such as the extracellular matrix or neighboring embedded endothelial cells and internal contractile forces of cancer cells may reveal new mechanically induced pathways promoting or increasing cancer cell invasion and transmigration through the endothelium of connective tissue. Finally, the force transmission and generation of the invasive cancer cells build the focus of many biophysical approaches studying cancer progression and the process of metastasis. However, the role of the endothelial cells in providing external forces regulating cancer cell transmigration and invasion still needs further investigation. In particular the role of the actin filaments, intermediate filaments and the microtubules in transmitting and generating forces are still the focus of current cellular biophysical research. In summary, our studies provided a novel role of the endothelium in initiating and promoting the invasion of cancer cells and we therefore postulate a mechanical role for endothelial cells in regulating cancer cell invasion and transmigration.

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