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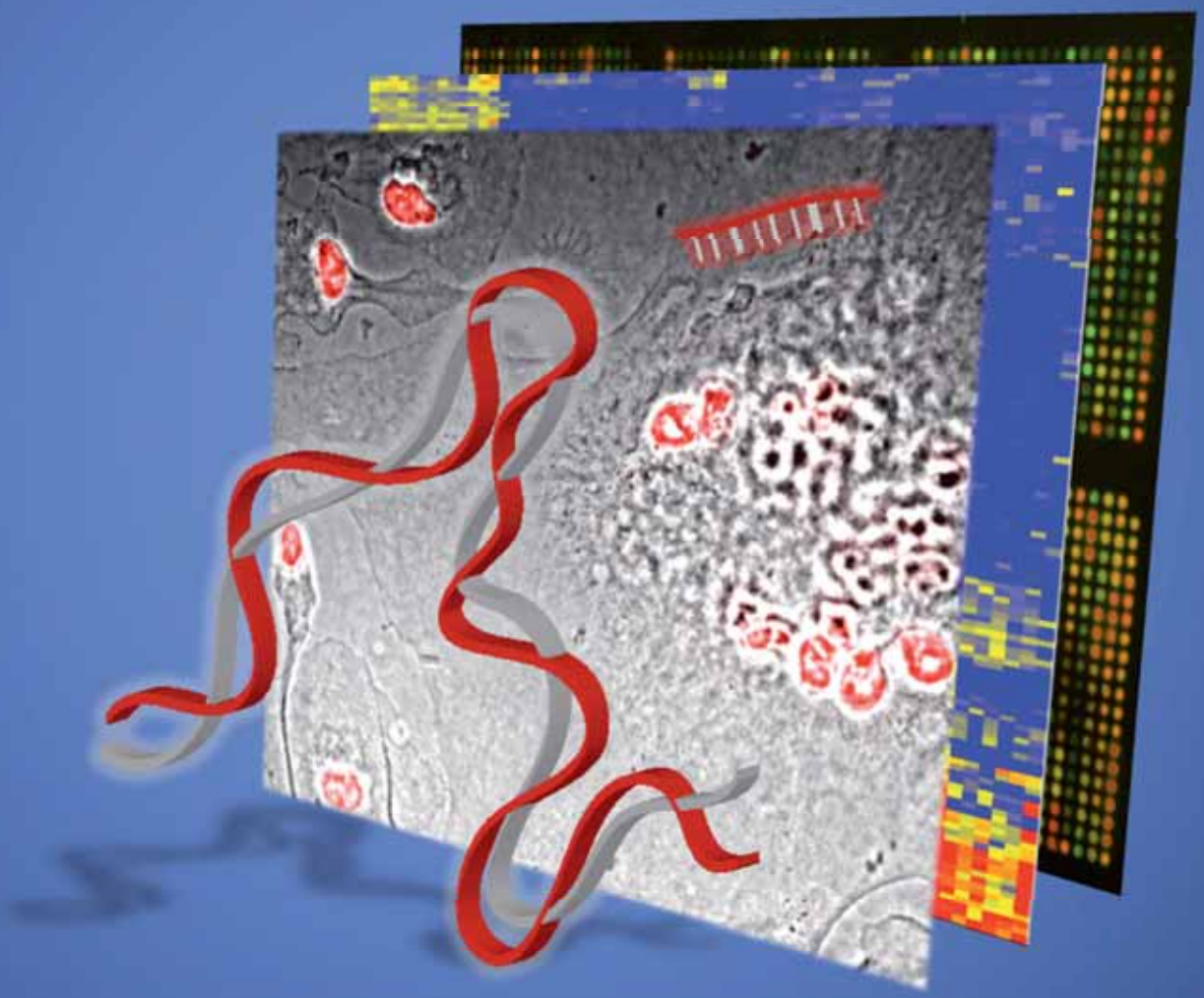

RNA and Disease

Young Scientist Meeting in Jena,
September 8-10, 2011

Deutsche Gesellschaft für Zellbiologie

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Cover image: Long non-coding RNAs and microRNAs are indispensable molecules in health and disease. Murine peritoneal macrophages fusing to multinucleated giant cells are visualized and the nucleus, a very important compartment for non-coding RNA function is counterstained in red using DRAQ5. Microarray and heat map images below display differentially expressed long non-coding RNAs in cancerous tissues. (Unpublished data kindly provided by Dr. Sven Diederichs, Molecular RNA Biology & Cancer group, DKFZ Heidelberg. Graphical design by Julia Winter and Tony Gutschner.)

We shall meet again ...

if your memory serves you well

RNA and Disease

The deadline for the next, the **12th Young Scientist Meeting** in Jena is approaching. This time it is for organizational reasons early, i.e. we accept applications until the 15th of July. Like in previous meetings, participants should present own data, as thereby the intensity of the discussion will receive an immense push, and indeed also the invited speakers will profit a lot from exposing themselves to the new generation of experts in the field. As you can see on the opposite page, thirteen renowned speakers will give presentation in addition to the organizers, Sven Diederichs and Dirk Grimm. In this issue, Sven presents a concept for the role that non-coding RNAs have in general and in developmental processes of higher organisms, hence they may have a potential as therapeutic agents (p. 6). Moreover, Dirk and colleagues have contributed a fantastic and extensive article on the importance of the RNAi machinery for human health (p. 8). These authors draw strong conclusions in favour of the role that both basic research and studies in patients will have in the future in order to facilitate their use in human medicine. In particular, to explore the networks that individual RNAis operate in will be of utmost importance to understand their functions and to dreive concepts for treatments in disease.

Physics of Cancer

In addition, another meeting is on display and open for registration. The "Physics of Cancer" is to be held in Leipzig, October 13th to 15th. The application deadline is August 31st (see p. 17). Also in this meeting we prefer participants that present own work, again in order to stimulate scientific discussion and exchange of ideas. As the organizers of this meeting, Josef Käs, Sarah Köster and myself wish to point out again something that has been advertised for the first meeting on the "Physics of Cancer" last year: The investigation of changes in physical properties of cells during malignant transformation is an emerging field in current cancer research and cell biology. Recent findings in this new field revealed that the biomechanical properties of tumour cells promote their growth as well as their invasion within the human body.
With this second meeting we indeed attempt to gather pioneering groups in the investigation of the physical mechanisms underlying cancer progression in order to initiate the formation of joint-force-groups from physics, cell and molecular biology as well as medicine. More scientific information on this topic will be found in the recent article in our Newsletter from the lab of Josef Käs: "Probing the physics of tumor cells from mechanical perspectives" (Mareike Zink et al. (2010) Cell News 36 (4), 17-21).

Annual Meeting 2012

As mentioned in the last *Newsletter*, the next Annual Meeting will be held in Dresden, March 21st to 24th, 2012. The first draft of the program has been assembled and now the chairs of the individual sessions have already contacted or are in the process of approaching potential speakers. The topics are balanced, however, the focus is on emerging fields with often, naturally, scientist in their early phase of research. Hence, we will have *Plenary Sessions* covering areas such a "Cell Adhesion and Migration", "Cell and Tissue Morphogenesis", "Nuclear Organization", "Cilia", and "Frontiers in Microscopy". The fifteen *Symposia* will deal with issues such as "Meiosis", "Mechanics of Cell Division", "Control of Cell and Organ Size" and "Evolution of the Cell" to name a few. The full program will be presented end of September both on the web and in *Cell News* 3/2011.

Future Meetings

We will be happy to receive suggestions for the Young Scientist Meeting 2012 (dgz@dkfz.de). The format is two and a half days with a speaker's schedule as for instance as can be seen in the program, for instance, of the 2008 meeting (Zellbiologie aktuell 2/2008). The meeting should be held at Carl-Zeiss in Jena, Thursday to Saturday. Otherwise we are open to every new topic. The list of all previous young scientist meetings is depicted below.

Previous Young Scientist Meetings

1. Gen Targeting – Genmanipulation – Gentherapie
2. Zelluläre Signaltransduktion
3. Zell-Zell und Zell-Matrix-Interaktionen während Entwicklung und Differenzierung
4. Embryonale und somatische Stammzellen in Grundlagenforschung und Medizin
5. Zellkernarchitektur
6. Cytoskeletal Dynamics
7. Vesicular Trafficking
8. Cell Biology of Cancer
9. Signaling Cascades in Development and Disease
10. Biology of Cell Division
11. Imaging Cell Migration
12. RNA and Disease

Hence, some of you may surely get an idea what kind of topic they might want to present and organize in the near future.

The *International DGZ Meeting* 2012 will be organized by Thomas Magin, Leipzig, and will focus on the various functions of the epidermis. Of course, early suggestions for a corresponding conference in 2013 are welcome. Incoming suggestions will be discussed at a board meeting of the DGZ in October this year.

Let me wish those of you that we will not see at the "Microtubule Spider Web" meeting in Potsdam, a pleasant summer and success with the many applications you surely will send out before the holiday season.

Harald Herrmann

12th Young Scientist Meeting of the German Society for Cell Biology (DGZ)

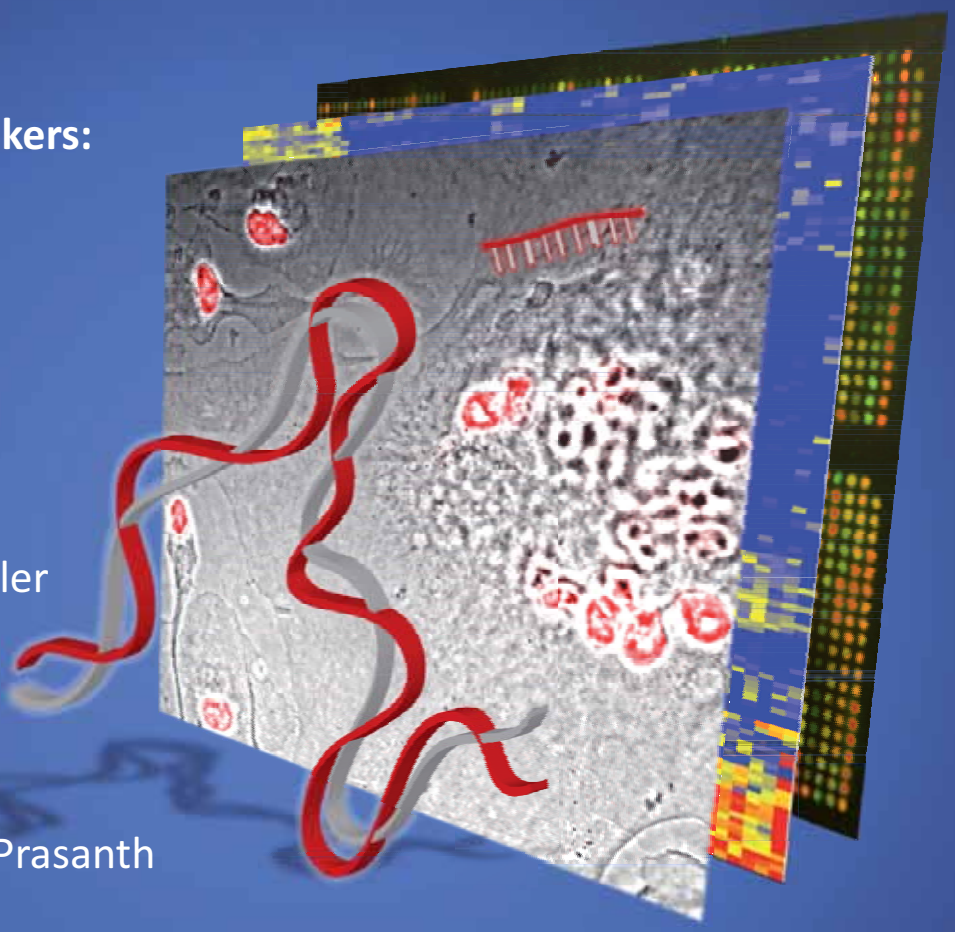
„RNA & DISEASE“

Jena, September 8th - 10th, 2011

Organizers: Sven Diederichs and Dirk Grimm

Confirmed Speakers:

Frank Slack
George Calin
Brian Brown
Reuven Agami
Utz Fischer
Ingrid Grummt
Ute Kothe
Markus Landthaler
Amaia Lujambio
Gunter Meister
Daniel Mertens
Dónal O‘Carroll
Kannanganattu Prasanth



Registration: 120 €, Students: 90 €, **free for DGZ members**.
Registration **includes accommodation and meals**. The number of participants is limited.

Master or Ph.D. students, postdocs and young group leaders are invited to apply for participation with an abstract. Participants will present their work as a poster or a short talk.
For more information and registration visit: **www.zellbiologie.de** .
Please send your application and questions by e-mail to the DGZ Office at: dgz@dkfz.de .

ZEISS

APPLICATION DEADLINE:
July 15th, 2011

DGZ

Non-protein-coding RNA – a whole new world of functional players in the cell?!

Sven Diederichs

According to the classical dogma of molecular biology, the genetic information is stored in the genome in the form of DNA, transcribed into the messenger molecule, the mRNA, that is then translated into a protein. Proteins were generally considered to be the only molecular machines and workhorses of the cell. In consequence, basic science as well as disease-oriented research has mostly focused on these cellular molecules in the past.

However, recent studies have revealed two important insights into the biology of RNAs that induce a paradigm shift and a significant extension of this dogma:

First, new technologies such as genome-wide tiling arrays or transcriptome deep sequencing approaches have revealed that a far greater fraction of the human genome is transcribed into RNA than previously anticipated. Researchers estimate that at least 70% of the human genome is transcribed while only about 1% - 2% are protein-encoding (Birney et al., 2007).

Second, more and more examples are accumulating that non-coding RNAs can execute important functions in the cell. On the one hand, very short non-coding RNAs, the microRNAs, play an important role in gene regulation (Winter et al., 2009) by targeting mRNAs for either degradation or translational inhibition (Meister et al., 2004). The tumor-suppressive or oncogenic role of many microRNAs as well as their frequent deregulation in many tumors allows a first glimpse on the striking role that non-coding RNAs could play in human malignancies (Esquela-Kerscher and Slack, 2006). On the other hand, novel examples of long non-coding RNAs - that do not form a homogeneous class such as microRNAs - are emerging that fulfill important functions in X chromosome dosage compensation (XIST & TSIX (Lee et al., 1999)), epigenetic regulation (Schmitz et al., 2010), chromatin remodeling (HOTAIR (Gupta et al., 2010; Rinn et al., 2007; Tsai et al., 2010)), Splicing and Metastasis (MALAT1 (Ji et al., 2003; Tripathi et al., 2010)) or the formation of RNA-protein complexes. Long non-coding RNAs can be highly conserved throughout evolution (Calin et al., 2007) and regulated by epigenetic mechanisms (Lujambio et al., 2010). This significantly broadens the range of functional long non-coding RNAs - also called ncRNA, lncRNA or lincRNA - beyond the functional RNAs involved in protein biosynthesis, such as ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) (Winkler et al., 2005) or small nucleolar RNA (snoRNA).

Taken together, the mammalian cell contains many more RNA molecules than previously anticipated and many of them might just await their discovery as functionally important molecules in development, health and disease.

At the same time, RNA is emerging as a potential therapeutic target as well as a potential therapeutic tool with the application of RNA interference as the most prominent example (Brown and Naldini, 2009; Grimm et al., 2010).

To shed light onto most recent developments in this rapidly emerging field, we invite young researchers to delve into the fascinating topic of **"RNA & Disease"** at the 12th DGZ Young Scientist Meeting in September 2011 in Jena. The main topics will cover microRNA function & biogenesis in disease, the function of long non-coding RNAs and their role in cancer, RNA metabolism & neurological disorders, snRNA function & splicing as well as RNA-based therapy options. The meeting will bring together national and international leaders in the field with young researchers interested in this emerging topic.

Citations:

Birney, E., Stamatoyannopoulos, J.A., Dutta, A., Guigo, R., Gingeras, T.R., Margulies, E.H., Weng, Z., Snyder, M., Dermitzakis, E.T., Thurman, R.E., et al. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* **447**, 799-816.

Brown, B.D., and Naldini, L. (2009). Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications. *Nat Rev Genet* **10**, 578-585.

Calin, G.A., Liu, C.G., Ferracin, M., Hyslop, T., Spizzo, R., Sevignani, C., Fabbri, M., Cimmino, A., Lee, E.J., Wojcik, S.E., et al. (2007). Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. *Cancer Cell* **12**, 215-229.

Esquela-Kerscher, A., and Slack, F.J. (2006). Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* **6**, 259-269.

Grimm, D., Wang, L., Lee, J.S., Schurmann, N., Gu, S., Borner, K., Storm, T.A., and Kay, M.A. (2010). Argonaute proteins are key determinants of RNAi efficacy, toxicity, and persistence in the adult mouse liver. *J Clin Invest* **120**, 3106-3119.

Gupta, R.A., Shah, N., Wang, K.C., Kim, J., Horlings, H.M., Wong, D.J., Tsai, M.C., Hung, T., Argani, P., Rinn, J.L., et al. (2010). Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* **464**, 1071-1076.

Ji, P., Diederichs, S., Wang, W., Boing, S., Metzger, R., Schneider, P.M., Tidow, N., Brandt, B., Buerger, H., Bulk, E., et al. (2003). MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene* **22**, 8031-8041.

Lee, J.T., Davidow, L.S., and Warshawsky, D. (1999). Tsix, a gene antisense to Xist at the X-inactivation centre. *Nat Genet* **21**, 400-404.

Lujambio, A., Portela, A., Liz, J., Melo, S.A., Rossi, S., Spizzo, R., Croce, C.M., Calin, G.A., and Esteller, M. (2010). CpG island hypermethylation-associated silencing of non-coding RNAs transcribed from ultraconserved regions in human cancer. *Oncogene* **29**, 6390-6401.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* **15**, 185-197.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., et al. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* **129**, 1311-1323.

Schmitz, K.M., Mayer, C., Postepska, A., and Grummt, I. (2010). Interaction of noncoding RNA with the rDNA promoter mediates recruitment of DNMT3b and silencing of rRNA genes. *Genes Dev* **24**, 2264-2269.

Tripathi, V., Ellis, J.D., Shen, Z., Song, D.Y., Pan, Q., Watt, A.T., Freier, S.M., Bennett, C.F., Sharma, A., Bubulya, P.A., et al. (2010). The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol Cell* **39**, 925-938.

Tsai, M.C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. *Science* **329**, 689-693.

Winkler, C., Eggert, C., Gradl, D., Meister, G., Giegerich, M., Wedlich, D., Laggerbauer, B., and Fischer, U. (2005). Reduced U snRNP assembly causes motor axon degeneration in an animal model for spinal muscular atrophy. *Genes Dev* **19**, 2320-2330.

Winter, J., Jung, S., Keller, S., Gregory, R.I., and Diederichs, S. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. *NatCell Biol* **11**, 228-234.

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12th DGZ Young Scientist Meeting "RNA and Disease"

September 8-10, 2011, Jena

Organizers: Sven Diederichs and Dirk Grimm

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

Registration fee:

EUR 120,00 for regular participants
EUR 90,00 for students
Free for DGZ members

Registration includes accommodation and meals.
The number of participants is limited.

Application:

Your letter of application should include in one single WORD file:

- Your name, e-mail address, institute, contact details and your status (DGZ member, student or regular participant)
- Your abbreviated CV (max. 1 page)
- Your abstract (max. 1 page incl. Title & Authors & Affiliations) in Arial, 12 p, 1.5 lines spacing

The file name should be your last and first name

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

Application Deadline: July 15th, 2011

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

Programme / Speakers:

Frank Slack: MicroRNAs in cancer

George Calin: Non-codingRNA principles for medical practice

Reuven Agami: microRNAs and regulatory RNA binding proteins in cancer

Brian D. Brown: Global sensing of miRNA activity

Sven Diederichs: Long non-coding RNA in Cancer

Utz Fischer: Defects in RNA metabolism as a cause of neurological disorders

Dirk Grimm: Synthetic AAV vectors as tools for safe shRNA expression & targeted miRNA regulation

Ingrid Grummt: Noncoding RNA targets chromatin modifying enzymes to regulatory gene sequences

Ute Kothe: H/ACA small ribonucleoproteins in health and disease

Markus Landthaler: Decoding post-transcriptional regulatory networks using high throughput approaches

Amaia Lujambio: The pervasive role of small RNAs in cancer

Gunter Meister: Analysis of Argonaute interactions in mammalian cells

Daniel Mertens: 13q14: miRs and more

Dónal O'Carroll: Title to be announced

Kannanganattu Prasanth: Role of a cancer-associated nuclear RNA in pre-mRNA splicing control

Perturbation of the cellular RNAi machinery in disease and therapy: Lessons learned and avenues paved

Stefan Mockenhaupt, Kathleen Börner and Dirk Grimm

Background

Dysregulation of RNA interference (RNAi) - an evolutionarily conserved essential cellular mechanism of gene and genome regulation - is increasingly found associated with cancers and several other human pathologies. Particularly characteristic is the frequent down-regulation of single or multiple miRNAs in tumor cells as compared to healthy tissues, a finding first reported in a seminal 2005 paper and subsequently validated in many further studies (1). Interestingly though, others have recently also reported opposite or mixed observations, such as concurrent down- and up-regulation of miRNA subsets upon infection of human cells with pathogenic viruses such as HIV-1 (2). Moreover, we and others found that ectopic stable overexpression of small RNAs can likewise readily and globally perturb the cellular RNAi machinery, as evidenced by aberrant endogenous miRNA expression, associated cytotoxicities and sometimes even animal fatalities (3). Notably, in many of these cases, amounts of mature miRNAs did not correlate with levels of primary miRNAs quantified in the same cell, implying that these phenomena are not solely due to alterations in miRNA expression but frequently also involve aberrant molecular processing.

For better understanding of this emerging important concept, we shall briefly recapitulate that humans possess at least 1424 miRNAs that are typically transcribed by RNA polymerase II promoters (Fig. 1, www.mirbase.org). The resulting pri(mary) miRNA - a stem-loop hairpin structure with flanking 5' and 3' sequences - is then trimmed by the RNase Drosha and its partner DGCR8 (together called the microprocessor) into a pre(cursor) miRNA which is next exported into the cytoplasm by the karyopherin Exportin-5. There, the pre-miRNA is further cropped by the enzyme Dicer together with its partner TRBP into roughly 22 nucleotides long mature miRNA which then associates with one of four related human Argonaute proteins (Ago-1 to -4) and becomes part of the RNA-induced silencing complex RISC. The latter finally binds to a target mRNA, typically in the 3' UTR at a position selected via partial homology to the RISC-incorporated miRNA, and inhibits its expression. The exact molecular mechanisms underlying this final step are still elusive, but accumulating evidence suggests that key players are the family of three related TNRC6 proteins which bind to Ago proteins and concurrently recruit mRNA decay factors (4). This might in turn partially explain reports that miRNA-mediated gene silencing involves target mRNA destabilization from both ends via decapping and deadenylation, albeit a flurry of other mechanisms reviewed elsewhere are likewise discussed, including changes in mRNA localization or repression of translation (5-10).

The fact that miRNAs control at least 30% of all human genes (potentially even more since over 60% of all human genes are predicted to contain miRNA binding sites in their 3'UTRs, plus miRNAs can also bind to open reading frames and promoters) readily explains why miRNAs are not only critically involved in a plethora of cellular key processes (e.g. differentiation, development and apoptosis) but also why their dysregulation is causally linked to human diseases. In view of their processing cascade outlined above, one can indeed imagine a myriad of complex molecular mechanisms leading to adverse dysregulation beyond "simple" alterations in pri-miRNA expression. In fact, we nowadays have evidence that miRNA processing can be altered at every single step along the biogenesis cascade, from Drosha trimming, Exportin-5 transport and Dicer cropping to RISC incorporation and target mRNA binding and regulation. Because the primary and review literature describing these findings has grown tremendously over the past five years (11-18), we will only briefly summarize the main models and conclusions in the next chapter. The main focus of the remainder of this article is then on most recently identified specific cases where the cellular RNAi machinery is globally perturbed, either in the course of human diseases (especially cancers or viral infections), or as a result of exogenous RNAi engagement via delivery and expression of RNAi gene therapy vectors. We will conclude with a brief discussion of the relevance of these findings for our comprehension of regulatory processes in human cells as well as with an outlook into the ne-

cessary steps to foster RNAi applications and clinical translation.

A myriad of cellular mechanisms controls maturation of individual miRNAs

As noted, the amazing complexity underlying intracellular processing of specific miRNAs has previously been reviewed comprehensively, including an excellent overview by Winter and Diederichs to which we refer for more detailed information (18). Here, suffice it to point out some remarkable mechanisms as representative examples for the overall intricacy, such as the set of feed-back loops controlling let-7 miRNA biogenesis. This miRNA is frequently down-regulated on the transcriptional level in tumor cells as well as post-translationally suppressed by the RNA binding protein Lin28 whose levels negatively correlate with let-7 during development. The mechanisms explaining this inverse correlation are very complex and include Lin28 binding to the terminal pre-let-7 loop, Lin28-mediated recruitment of the non-canonical poly(A) polymerase TUT4 (which adds a uridine tail to pre-let-7 and thus renders it unavailable for Dicer cleavage), Drosha and Dicer repression by Lin28 and finally also let-7-induced repression of c-Myc which in turn up-regulates Lin28 (19-24).

Similar complex events were also reported for example for miR-18a (one of six miRNAs in the miR~17-92 cluster) whose maturation is facilitated by the hnRNPA1 protein which binds to the conserved loop of miR-18a and thereby induces a structural change creating a more favorable Drosha/DGCR8 binding and cleavage site (25; 26). A third example are let-7a and miR-206 whose Drosha/DGCR8- and Dicer-mediated processing is synergistically promoted by binding of the KSRP protein to G-rich regions within their terminal loops (26; 27). Finally also noteworthy, some miRNAs utilize non-canonical miRNA processing pathways and thus underlie completely different and unique regulatory mechanisms, such as miR-451 which bypasses Dicer cleavage and is instead directly loaded into Ago-2 for further processing, or the ac-pre-miRNAs discovered by Diederichs and Haber that are pre-processed by Ago-2 prior to Dicer-mediated trimming (28-30).

Besides differential processing, miRNAs can also be regulated via their stability, e.g. miR-122 which in human liver cells is stabilized via GLD-2-mediated 3' monoadenylation, or in male late-stage germ cells via the DNA/RNA-binding protein translin (31; 32). Mo-

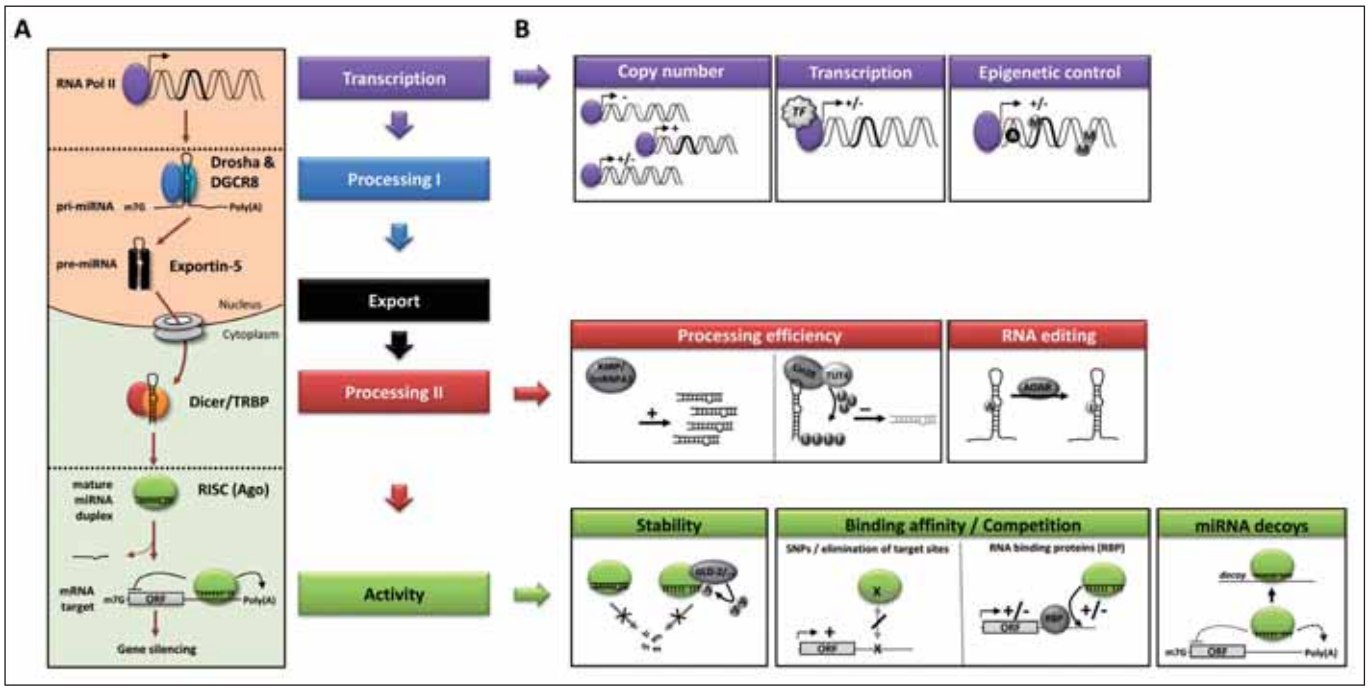

Figure 1: The canonical RNAi pathway in humans and representative examples for its regulation. *(A) Shown is a scheme of a cell (nucleus in orange and cytoplasm in green) along with the individual steps of miRNA biogenesis, starting with miRNA transcription (typically via RNA polymerase II), followed by a first processing in the nucleus, export into the cytoplasm and second processing there, and finally culminating in miRNA loading into RISC and ultimate target mRNA binding and silencing. See text for more details. (B) As also described more comprehensively in the text, each step in this cascade can be regulated individually, including control on the transcriptional level (shown in purple; e.g. via miRNA copy number alterations, different promoter strengths or epigenetic control mechanisms), on the processing level (shown in red; e.g. via miRNA binding proteins that enhance or inhibit miRNA processing, or via miRNA editing (not detailed in the main text)), and finally also on the RISC/activity level (shown in green; e.g. via regulation of miRNA stability, alterations in or competition with target mRNA binding, or sequestration of active miRNAs by decoy mRNAs (such as pseudogenes)).*

reover, miRNAs can be sequestered and thus inactivated by pseudogenes that act as decoys containing multiple miRNA binding sites in their 3'UTR, e.g. the PTEN/PTENP1 (pseudo)genes (33). Along these lines, miRNA binding to the genuine target mRNA can additionally be blocked by competing proteins, such as in the case of the AU-rich-element binding protein HuR that binds to the CAT-1 mRNA 3'UTR and thus prevents its regulation by miR-122 (34). Further intriguing examples are the RNA-binding Dnd1 protein which derepresses mRNAs with U-rich miRNA binding sites, or the PUM1 protein which binds to the p27 mRNA 3'UTR and induces local changes in mRNA structure that facilitate binding of miR-221/-222 (35; 36).

As a final large set of examples for specific RNAi alterations, we briefly wish to mention the modulation of individual RNAi proteins while again referring to more comprehensive recent reviews for details (see references above). One remarkable recently discovered example is the intricate auto-regulatory loop by which Drosha activity is regulated by DGCR8 and vice versa. In this loop, DGCR8 stabilizes Drosha through direct protein-protein interaction, while Drosha causes DGCR8 mRNA degradation by cleaving two miRNA-like hairpins in this mRNA (37). Whether this double-negative feed-back loop is specific to the Drosha-DGCR8 pair, or whether such miRNA-like hairpin-containing mRNAs compete with endogenous genuine miRNAs for processing, are interesting questions for future research. Akin to Drosha and DGCR8, Dicer and TRBP are another pair of RNAi factors which are co-regulated in cells. Here, TRBP is controlled by the MAPK pathway in which it becomes phosphorylated at four serines, leading to its up-regulation and in turn an increase in Dicer levels, likely via TRBP-mediated Dicer protein stabilization (38). As a final important example, Ago-2 is also regulated post-translationally, especially through phosphorylation or hydroxylation. Notably, the functional consequences of these Ago-2 modifications remain partly elusive, exemplified by its phosphorylation at serine 387 that facilitates Ago-2 localization to P-bodies without known physiological significance (especially since P-bodies do not seem mandatory for RNAi) (39). In contrast, Meister's group postulated that Ago-2 phosphorylation at tyrosine 529 (located in the small RNA 5' end-binding pocket in the Ago MID domain) might functionally act as a molecular switch promoting or inhibiting small RNA loading into Ago proteins (40). Yet again, important questions remain open, such as which kinase mediates this phosphorylation or whether the phosphate is directly transferred between small RNA and Ago protein. Similar uncertainties are associated with Ago protein modification via hydroxylation at proline 700. While reported to stabilize Ago-2, it had no effect on its target mRNA slicing (i.e. cleavage) capability, leaving its physiological relevance unclear (41).

The RNAi machinery is frequently globally dysregulated in human diseases

As stated initially, the main focus of this article is on the rapidly emerging reports that human diseases - especially cancers, neurological disorders and viral infections - as well as RNAi-based gene therapies are associated with global perturbations of the cellular RNAi machinery. As the literature on this new research area has already become very extensive, we can however again only pick selected representative examples and only reference a few respective original publications. The main findings are also summarized in Fig. 2A/B.

As the first essential factor in the miRNA processing cascade, it may not surprise that Drosha is frequently dysregulated in various cancers; yet the kind of alteration (up- or down-regulation) as well as the mechanism seem to vary, and no clear pattern or value as diagnostic or prognostic marker has yet emerged. For instance, a series of papers reported Drosha up-regulation in different human cancers, e.g. esophageal squamous cell carcinoma, epithelial skin cancers, cervical squamous cell carcinoma or ovarian serous adenocarcinomas, to name just a few (42-44). Interestingly, the increase in Drosha often correlated with clinico-pathological tumor characteristics, in a sense that high Drosha abundance was predictive for progressive disease, enhanced tumor invasion and overall poor prognosis. At least in some cases, there was evidence that Drosha elevation was due to genetic copy number gains. What remained puzzling and counter-intuitive though are observations that many miRNAs were down- instead of up-regulated, implying their potential differential control via unknown mechanisms.

Strikingly, a large number of papers also reported the opposite, i.e. Drosha decreases in human cancers or no significant changes at all, together exemplifying the intricacies of cellular RNAi pathways and the gaps in our understanding of their regulation. These findings were again made in different tumors, including some in which others had noted Drosha up-regulations. Examples are ovarian and breast cancers where inherent Drosha down-regulations occasionally seemed to correlate with suboptimal cytoreductive surgery and reduced median overall survival (45; 46). Moreover, others found no relationship between Drosha and patient survival, but rather speculated that the miRNA fluctuations observed in various tumors are due to DNA copy number alterations and/or epigenetic silencing, rather than global perturbation of the miRNA processing machinery.

Finally, Drosha dysregulation was also noted in other human diseases, most notably neuropathologies such as schizophrenia or Huntington's disease (47; 48). Again, no clear pattern has yet emerged regarding Drosha levels and patient outcome, and the correlation between Drosha perturbation and miRNA dysregulation also remained obscure. Yet it is clear that miRNAs play a key role

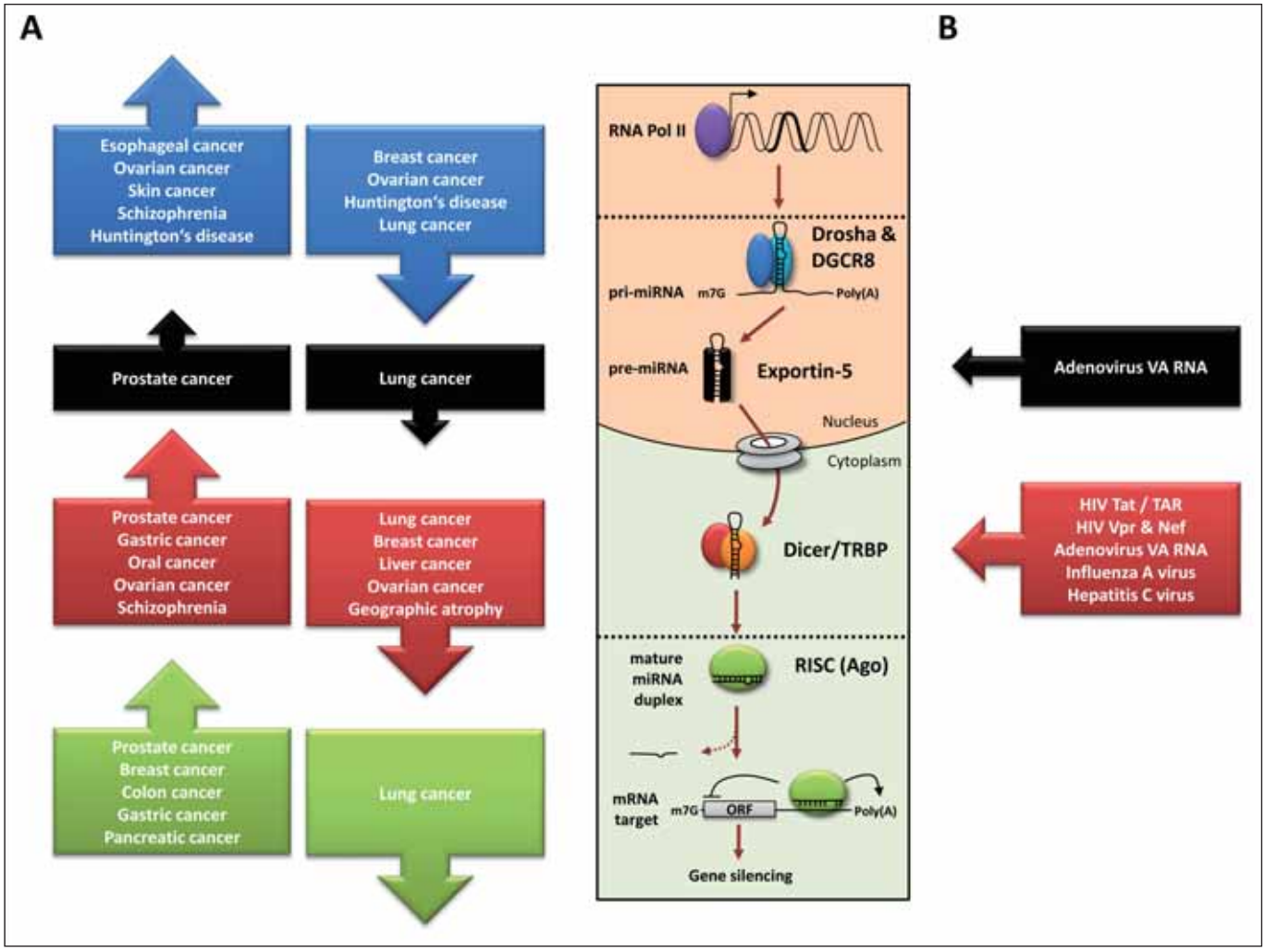

Figure 2: Perturbations of the human RNAi pathway in cancer, neuropathologies and viral infections. *(A) Shown are selected examples of human cancers or neuropathologies in which main RNAi factors/steps are perturbed (up- or down-regulated, as indicated by the arrows). Depicted in blue are alterations at the Drosha/DGCR8 level, in black at the Exportin-5 level, in red at the Dicer/TRBP level and in green at the RISC/Ago level. Note that this list is not comprehensive, and that the literature is controversial in several cases (see text and references therein for more details). (B) Depicted are some of the best studied examples for human viruses and their components that likewise perturb the RNAi pathway at the Exportin-5 (black) or Dicer/TRBP (red) levels.*

in human neuropathologies, and it is likely that dynamic alterations in the cellular RNAi machinery are critically involved as well.

Akin to Drosha, its partner DGCR8 was also found to be dysregulated in some cancers and other diseases, which is unsurprising considering the mentioned tight co-regulation of these two factors. Examples are salivary gland pleiomorphic adenomas or schizophrenia in which DGCR8 was up-regulated, potentially due to low copy repeat-induced micro-duplications and resulting elevated gene dosages (49). Still, Drosha and DGCR8 alterations not always coincided, and their association with probability of survival or prognosis also sometimes varied, suggesting differential control of these two factors and of downstream events in the cell at least in some cases.

Unlike Drosha and DGCR8, there are few data on disease-related Exportin-5 perturbation thus far, except for isolated reports on its up-regulation e.g. in prostate cancers (correlating with increased metastasization) or its down-regulation in certain stages of lung cancer (50; 51). Dicer, on the other hand, is probably the currently best studied member of the miRNA processing machinery regarding perturbation in the context of human pathologies. Hallmark findings were made in various cancers such as prostate adenocarcinoma where high Dicer levels were prognostic markers for advanced disease and poor outcome (52). Curiously, it was the opposite in other cancers such as lung tumors where low Dicer levels (potentially caused by loss of heterozygosity of chromosome 14 where the Dicer gene resides) correlated with reduced probability of patient survival (53; 54). To

make matters even more complex, Dicer was found nearly equally up- or down-regulated in head and neck cancers, with both cases correlating with poorer disease-specific survival (55). Mechanistically, one interesting model postulated Dicer mRNA regulation via the let-7 miRNA family as one of the underlying mechanisms which could in fact explain the occasionally observed discrepancies between Dicer mRNA and protein levels in several studies. Besides, Dicer perturbations were also noted in further tumors such as leukemias or skin, gastric and liver cancers. Yet again, Dicer was either up- or down-regulated in these various tumors, and the relevance for disease outcome also often remained open, leaving a flurry of interesting questions and topics for future studies. This is also true for the largest set of tumors studied with regard to Dicer perturbation to date, breast and ovarian cancers, where the controversy concerning up- or down-regulation and correlation with clinico-pathological parameters is particularly pronounced. One especially interesting aspect here is the potential Dicer association with estrogen receptor status as well as with the expression or activity of specific miRNAs which in turn control Dicer, providing intriguing clues regarding possible molecular mechanisms certainly worth studying further (56-58).

Finally interesting to note is a recent study reporting a role for Dicer down-regulation in geographic atrophy (a type of age-related macular degeneration) (59). Here, Dicer depletion in the retinal pigment epithelium seems to result in an increase in Alu repeats which in turn induces epithelium death (via unknown mechanisms). This finding of Dicer's role as a cyto-protective miRNA-independent cellular surveillance factor raises important questions and theories and implies that our view of key RNAi factors in human disease is still far from complete.

Last but not least, Ago-2 was also perturbed in different cancers, including breast, ovarian, prostate and colon tumors, albeit the literature is not yet comprehensive (56; 57; 60). Interestingly, in some cases, Ago-2 expression showed an anti-correlation with Dicer and with estrogen receptor status (in breast and ovarian cancers), highlighting that cancer pathology may generally depend on alterations of multiple RNAi factors and thus raising caution in over-interpreting data on individual miRNAs or RNAi components. Also, the notion that only Ago-2 was dysregulated amongst the four human Ago proteins may hint at a role outside the miRNA pathway or exemplify the particular function of Ago-2 in miRNA processing. Finally, the initial mechanistic insights gained in these first studies (e.g. recent findings that Ago-2 may be post-translationally stabilized via the EGFR/MAPK pathway) create considerable hope to find new clinical targets and hence effective strategies to treat female cancers and other devastating diseases that causally involve RNAi perturbations.

RNAi pathways are likewise frequently affected by viral infections

To date, a vast literature already describes mutual interactions of human pathogenic viruses with cellular RNAi, including virus-induced dysregulation of individual miRNAs which control host dependency or restriction factors and are accordingly up- or down-regulated by the virus, respectively. Further examples are exploitation of host-encoded miRNAs for regulation of viral gene expression, or even the embedding of miRNAs within the viral genome which in turn regulate host gene expression in favor of the virus (61; 62).

Most interesting for the present article are however notions that many viruses moreover encode suppressors of RNAi silencing that qualitatively or quantitatively perturb or block the host RNAi machinery (Fig. 2B). One notable first example is HIV-1 whose Tat protein may inhibit Dicer activity and hence block Dicer-dependent RNAi triggers, i.e. ectopic shRNAs as well as cellular miRNAs, albeit this was debated by others (63; 64). In addition, HIV Vpr and Nef proteins may also perturb Dicer expression in monocyte-derived macrophages, potentially via dysregulation of a specific miRNA which in turn suppresses Dicer mRNA (65). Furthermore, the HIV TAR region (a stem-bulge-loop RNA structure within HIV-1 transcripts) can sequester TRBP to shunt it away from Dicer/RISC and thus block RNAi (66). Noteworthy, all these conclusions were reached in artificial cell culture systems, leaving the biological relevance in the context of an actual HIV-1 infection unclear to date.

Similarly to HIV, human Adenovirus can also block cellular RNAi machinery via multiple mechanisms, including Exportin-5 saturation and Dicer inhibition by the abundantly expressed adenoviral VA RNAs (67-69). Interestingly, the latter are processed into functional siRNAs in infected cells, but whether or not they serve any role for the virus during infection remains open to date. One study in fact suggested that their main function is to block Exportin-5 which then results in a concurrent reduction in Dicer levels, thus amplifying the inhibitory effect of Adenovirus infection on host RNAi (70). Besides, it should only briefly be mentioned that other viruses have likewise evolved intricate means to interfere with host RNAi, such as influenza A or hepatitis C virus which target Dicer. For more insight into this exciting and rapidly emerging field of research, we refer the reader to more specialized recent reviews (61; 62). Finally interesting to note are latest concepts according to which deliberate inhibition of host RNAi mechanisms can be exploited to improve the production of recombinant gene transfer vectors derived from naturally occurring viruses (71). At this point, we can readily anticipate that many further molecular mechanisms by which viruses perturb human RNAi await their discovery, and that we will continue to draw many additional benefits from research in this new and prospering area.

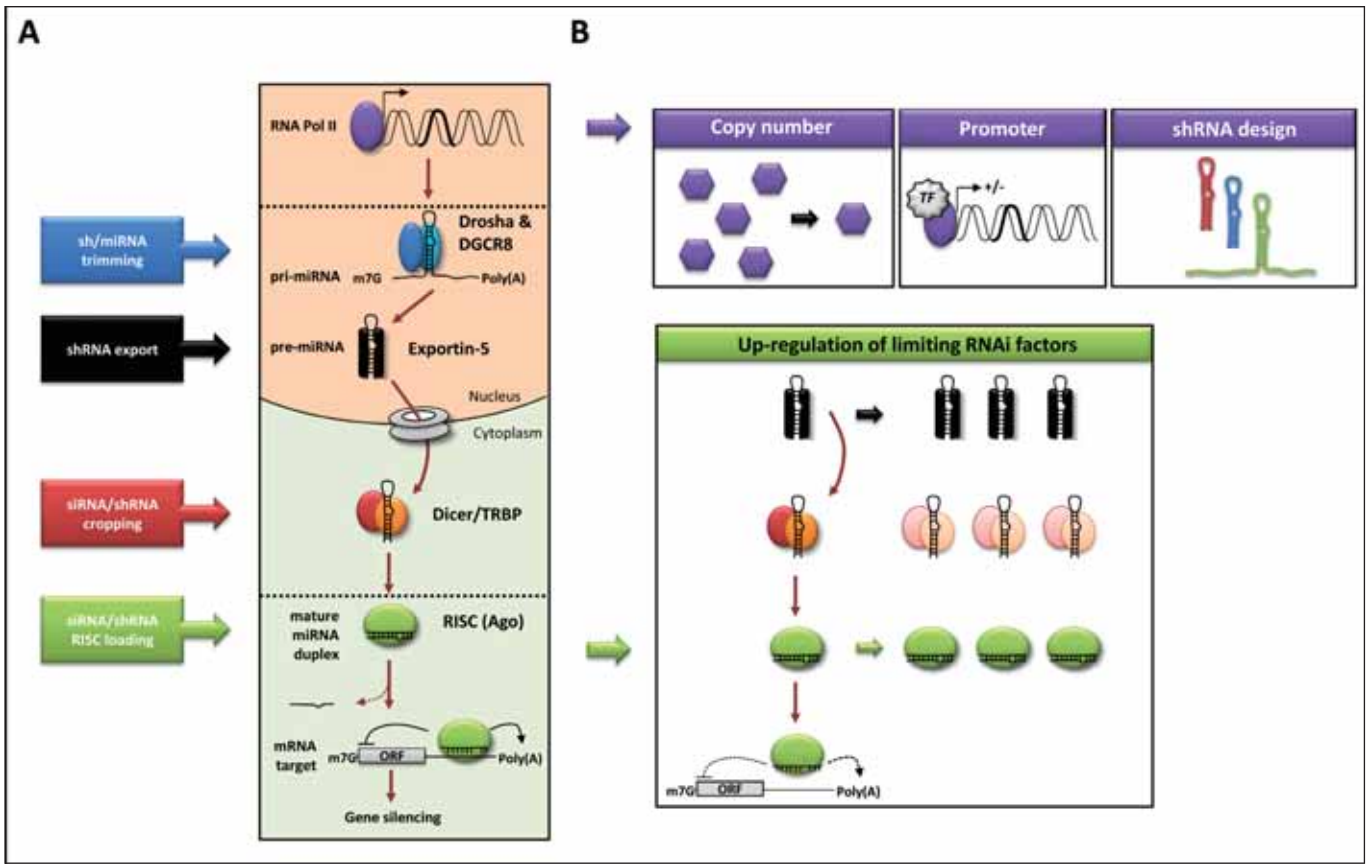

Figure 3: Saturation of the RNAi pathway by ectopic RNAi triggers and strategies to alleviate this risk. *(A) Depicted are possible means to unintentionally saturate and/or perturb the human RNAi pathway with ectopically delivered or expressed artificial RNAi triggers. The box in blue indicates the hypothetical risk to overload Drosha/DGCR8 with siRNAs expressed in a hybrid sh/miRNA backbone. The other three boxes depict the risk of saturating downstream steps (black: Exportin-5; red: Dicer/DGCR8; green: RISC/Ago) with shRNAs or again with hybrid sh/miRNAs. Note that RISC/Ago can be saturated with any artificial RNAi trigger, including siRNAs. (B) Representation of selected strategies to potentially alleviate the events shown in (A) and to accordingly improve the safety (and efficiency) of RNAi applications or therapies. As discussed in the text, these strategies include reduction in RNAi vector copy number, use of moderate promoters, selection of shRNAs with low inherent toxicity and possibly their expression from a miRNA scaffold (albeit this might increase the risk of Drosha/DGCR8 saturation, see (A)). Moreover, at least for selected applications such as cancer gene therapies that require fast and potent RNAi responses, it may be promising to concurrently up-regulate rate-limiting RNAi factors, particularly Exportin-5 and Ago-2.*

RNAi-based gene therapies can unintentionally perturb host RNAi

Having just noted how deliberate inhibition of host RNAi pathways may be a novel means to enhance viral vector production, it is curious that viral vectors can vice versa adversely interfere with cellular RNAi mechanisms and thereby cause toxicities (Fig. 3A). The first respective observation was made in 2006 by Grimm and colleagues who found that livers of adult mice treated with high doses of adeno-associated viral (AAV) vectors expressing shRNAs against hepatitis B virus (HBV) or other genes exhibited signs of massive hepatotoxicity (3). The fact that nearly half of the various viral shRNA vectors tested induced severe in vivo morbidity and even mortalities implied that the underlying effect was non-specific. Indeed, thorough analyses revealed an interference with hepatic miRNA expression, potentially due to shRNA dose-dependent saturation of cellular Exportin-5. Notably, similar shRNA-specific adverse saturation or competition events involving Exportin-5 plus other RNAi components (Dicer, TRBP) were also observed by others in cell culture or in brains of mice (72-74). Moreover, two recent studies recapitulated in vivo shRNA toxicities and fatalities upon injection of potent shRNA-expressing AAV vectors into brains of mice or rats, at least in once case again involving dysregulation of cellular miRNAs (75; 76). Also, another group reported that two common lentiviral shRNA expression libraries caused substantial shRNA dose-dependent perturbations of miRNA expression in a liver cell line resulting in numerous false-positive hits in screens for HCV host factors (77). Finally, a comprehensive meta-analysis of over 150 previously published siRNA or shRNA transfection experiments concluded that competition and saturation between extrinsic and intrinsic RNAi triggers are common and fre-

quently significantly impact the outcome of in vitro RNAi screens (78).

Altogether, the dire results of these studies clearly underscore the need to fully unravel the cellular mechanisms underlying perturbation of host RNAi and to develop novel safer strategies for future RNAi gene therapies and other applications (Fig. 3B). This is further exemplified by our recent conclusion that even marginal shRNA cytotoxicities suffice to trigger severe adverse events, based on our observation of accelerated tumorigenesis in livers of c-Myc-expressing mice treated with low doses of shRNA-expressing AAV vectors (79). Luckily, we already have some insight into the key molecular RNAi processes that are rate-limiting and hence particularly prone to saturation by ectopic RNAi triggers, including nuclear shRNA or miRNA export via Exportin-5. Noteworthy again are also the above mentioned findings that Exportin-5 and Dicer levels might be coupled in mammalian cells, providing further insights into the molecular outcomes of Exportin-5 saturation (70). In addition, we and others have recently identified Ago-2 as another major limiting factor in cultured cells as well as in murine livers (80; 81). Accordingly, its transient or stable (using AAV vectors) overexpression not only enhanced and extended RNAi but moreover also alleviated in vivo toxicities (81). Nonetheless, the fact that alleviation was only partial suggests that more work is needed to fully unravel and understand the cellular processes governing RNAi toxicities.

Until then, one can already envision multiple avenues to enhance and improve the safety and efficiency of RNAi applications in vitro or in vivo (Fig. 3B). One is the just mentioned deliberate up-regulation of limiting cellular factors, particularly Exportin-5 and Ago-2, albeit we currently lack long-term in vivo data on potential side effects. Still, this strategy to temporarily boost RNAi effects could be especially useful in tumor therapies where fast and strong responses are a top priority. An alternative strategy is to lower vector and hence shRNA doses to a minimum avoiding toxicities yet maintaining efficacy. This approach is equally promising, particularly when combined with selection for shRNAs that are inherently less toxic (for reasons still unclear, making this process empirical), as shown in our two recent studies where we obtained over one year silencing of HBV in HBV-transgenic mice using a preselected shRNA driven from either a weak RNA polymerase III or a likewise moderate liver-specific RNA polymerase II promoter (74; 81). Finally, a third potential strategy is to express therapeutic RNAi sequences from a miRNA scaffold which may slow RISC loading and hence also prevent saturation-induced toxicities, as speculated based on data in the mouse brain (72; 82; 83). However, as generation of such hybrid vectors is still not straight-forward and because resulting constructs vary in their shRNA expression and thus efficiency, this particular strategy clearly requires more fundamental work before it becomes routinely applicable.

Lessons learned thus far and new avenues ahead

These times are certainly exciting as we not only continuously make substantial progress in our understanding of basic cellular RNAi processes and related regulatory events, but at the same time we witness the ongoing translation of RNAi strategies into the clinics. The major lesson that one can learn from the findings reviewed above is that these two paths - intense basic research as well as concurrent study in animals and patients - are mutually intertwined and depend on each other. For us as biologists or doctors, this poses major opportunities yet also some challenges: From a basic research standpoint, the field will likely advance more rapidly if we intensify our efforts to appreciate the human RNAi machinery as an intricate and interwoven cellular network, rather than a collection of autonomous RNAs or proteins. By looking at RNAi from a systems biology perspective, where the whole is greater than the sum of its parts, and by opening our minds to other disciplines such as bioinformatics and modeling, we may indeed much faster become able to grasp and molecularly explain the adverse phenomena noted above.

The same is true for the medical or applied standpoint, where we also need to better appreciate human RNAi networks in all their intricacy rather than regard them as a collection of single molecules that one can presumably modulate and therapeutically correct at will. Instead, perturbation of one component - (mi) RNA or protein - will likely affect many other players in the RNAi pathway as well and hence inevitably cause global effects on the cell and organism. Therefore, we wish to raise caution for researchers or doctors aiming at tinkering with a single miRNA; despite some promising recent data that this may have therapeutic effects in animals, the long-term outcomes of such focused strategies that disregard the whole cellular picture remain largely unclear at this point.

Fortunately, looking ahead, several new avenues to improve the safety of RNAi have already been taken, and many further may lay in front of us. As outlined above, ongoing research has already provided evidence for the promise to apply marginal vector and shRNA doses, to express shRNAs from safer scaffolds, or to counter-act toxicities by up-regulating rate-limiting factors (see also Fig. 3B again). In addition, others suggested the use of small compounds to regulate and potentially correct human RNAi mechanisms, or reported protein tethering strategies that function independently of small RNA-based RNAi triggers and hence may overcome part of the cytotoxicities. As we expect that these and many other new avenues will be pursued and explored at the same breathtaking speed like all other RNAi research over the last decade, we are well optimistic that truly safe yet effective RNAi applications and therapies will be implemented in the not-too-distant future.

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References

1. Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., et al. (2005). MicroRNA expression profiles classify human cancers. *Nature* **435**, 834-838.
2. Triboulet, R., Mari, B., Lin, Y. L., Chable-Bessia, C., Bennasser, Y., et al. (2007). Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* **315**, 1579-1582.
3. Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., et al. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537-541.
4. Eulalio, A., Tritschler, F. & Izaurralde, E. (2009). The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing. *RNA* **15**, 1433-1442.
5. Filipowicz, W., Bhattacharyya, S. N. & Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* **9**, 102-114.
6. Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215-233.
7. Eulalio, A., Huntzinger, E. & Izaurralde, E. (2008). Getting to the root of miRNA-mediated gene silencing. *Cell* **132**, 9-14.
8. Fabian, M. R., Sonenberg, N. & Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* **79**, 351-379.
9. Huntzinger, E. & Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet* **12**, 99-110.
10. Pillai, R. S., Bhattacharyya, S. N. & Filipowicz, W. (2007). Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol* **17**, 118-126.
11. Carthew, R. W. & Sontheimer, E. J. (2009). Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136**, 642-655.
12. Davis, B. N. & Hata, A. (2009). Regulation of MicroRNA Biogenesis: A miRiad of mechanisms. *Cell Commun Signal* **7**, 18.
13. Ding, X. C., Weiler, J. & Grosshans, H. (2009). Regulating the regulators: mechanisms controlling the maturation of microRNAs. *Trends Biotechnol* **27**, 27-36.
14. Heo, I. & Kim, V. N. (2009). Regulating the regulators: posttranslational modifications of RNA silencing factors. *Cell* **139**, 28-31.
15. Kim, V. N., Han, J. & Siomi, M. C. (2009). Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* **10**, 126-139.
16. Siomi, H. & Siomi, M. C. (2010). Posttranscriptional regulation of microRNA biogenesis in animals. *Mol Cell* **38**, 323-332.
17. Trabucchi, M., Briata, P., Filipowicz, W., Rosenfeld, M. G., Ramos, A., et al. (2009). How to control miRNA maturation? *RNA Biol* **6**, 536-540.
18. Winter, J., Jung, S., Keller, S., Gregory, R. I. & Diederichs, S. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* **11**, 228-234.
19. Heo, I., Joo, C., Cho, J., Ha, M., Han, J., et al. (2008). Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol Cell* **32**, 276-284.
20. Heo, I., Joo, C., Kim, Y. K., Ha, M., Yoon, M. J., et al. (2009). TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell* **138**, 696-708.
21. Newman, M. A., Thomson, J. M. & Hammond, S. M. (2008). Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. *RNA* **14**, 1539-1549.
22. Piskounova, E., Viswanathan, S. R., Janas, M., LaPierre, R. J., Daley, G. Q., et al. (2008). Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28. *J Biol Chem* **283**, 21310-21314.
23. Rybak, A., Fuchs, H., Smirnova, L., Brandt, C., Pohl, E. E., et al. (2008). A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. *Nat Cell Biol* **10**, 987-993.
24. Wulczyn, F. G., Smirnova, L., Rybak, A., Brandt, C., Kwidzinski, E., et al. (2007). Post-transcriptional regulation of the let-7 microRNA during neural cell specification. *FASEB J* **21**, 415-426.
25. Guil, S. & Caceres, J. F. (2007). The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. *Nat Struct Mol Biol* **14**, 591-596.
26. Michlewski, G. & Caceres, J. F. (2010). Antagonistic role of hnRNP A1 and KSRP in the regulation of let-7a biogenesis. *Nat Struct Mol Biol* **17**, 1011-1018.
27. Trabucchi, M., Briata, P., Garcia-Mayoral, M., Haase, A. D., Filipowicz, W., et al. (2009). The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. *Nature* **459**, 1010-1014.
28. Cheloufi, S., Dos Santos, C. O., Chong, M. M. & Hannon, G. J. (2010). A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature* **465**, 584-589.
29. Cifuentes, D., Xue, H., Taylor, D. W., Patnode, H., Mishima, Y., et al. (2010). A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science* **328**, 1694-1698.
30. Diederichs, S. & Haber, D. A. (2007). Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell* **131**, 1097-1108.
31. Katoh, T., Sakaguchi, Y., Miyauchi, K., Suzuki, T., Kashiwabara, S., et al. (2009). Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. *Genes Dev* **23**, 433-438.
32. Yu, Z. & Hecht, N. B. (2008). The DNA/RNA-binding protein, translin, binds microRNA122a and increases its in vivo stability. *J Androl* **29**, 572-579.
33. Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W. J., et al. (2010). A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* **465**, 1033-1038.
34. Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I. & Filipowicz, W. (2006). Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. *Cold Spring Harb Symp Quant Biol* **71**, 513-521.
35. Kedde, M., Strasser, M. J., Boldajipour, B., Oude Vrielink, J. A., Slanchev, K., et al. (2007). RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell* **131**, 1273-1286.
36. Kedde, M., van Kouwenhove, M., Zwart, W., Oude Vrielink, J. A., Elkon, R., et al. (2010). A Pumilio-induced RNA structure switch in p27-3' UTR controls miR-221 and miR-222 accessibility. *Nat Cell Biol* **12**, 1014-1020.
37. Han, J., Pedersen, J. S., Kwon, S. C., Belair, C. D., Kim, Y. K., et al. (2009). Posttranscriptional crossregulation between Drosha and DGCR8. *Cell* **136**, 75-84.
38. Paroo, Z., Ye, X., Chen, S. & Liu, Q. (2009). Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling. *Cell* **139**, 112-122.
39. Zeng, Y., Sankala, H., Zhang, X. & Graves, P. R. (2008). Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies. *Biochem J* **413**, 429-436.
40. Rudel, S., Wang, Y., Lenobel, R., Korner, R., Hsiao, H. H., et al. (2010). Phosphorylation of human Argonaute proteins affects small RNA binding. *Nucleic Acids Res* **39**, 2330-2343.
41. Qi, H. H., Ongusaha, P. P., Myllyharju, J., Cheng, D., Pakkanen, O., et al. (2008). Prolyl 4-hydroxylation regulates Argonaute 2 stability. *Nature* **455**, 421-424.
42. Merritt, W. M., Lin, Y. G., Han, L. Y., Kamat, A. A., Spannuth, W. A., et al. (2008). Dicer, Drosha, and outcomes in patients with ovarian cancer. *N Engl J Med* **359**, 2641-2650.
43. Muralidhar, B., Goldstein, L. D., Ng, G., Winder, D. M., Palmer, R. D., et al. (2007). Global microRNA profiles in cervical squamous cell carcinoma depend on Drosha expression levels. *J Pathol* **212**, 368-377.
44. Sand, M., Gambichler, T., Skrygan, M., Sand, D., Scola, N., et al. (2010). Expression levels of the microRNA processing enzymes Drosha and dicer in epithelial skin cancer. *Cancer Invest* **28**, 649-653.
45. Dedes, K. J., Natrajan, R., Lambros, M. B., Geyer, F. C., Lopez-Garcia, M. A., et al. (2011). Down-regulation of the miRNA master regulators Drosha and Dicer is associated with specific subgroups of breast cancer. *Eur J Cancer* **47**, 138-150.
46. Tchernitsa, O., Kasajima, A., Schafer, R., Kuban, R. J., Ungethum, U., et al. (2010). Systematic evaluation of the miRNA-ome and its downstream effects on mRNA expression identifies gastric cancer progression. *J Pathol* **222**, 310-319.
47. Beveridge, N. J., Gardiner, E., Carroll, A. P., Tooney, P. A. & Cairns, M. J. (2010). Schizophrenia is associated with an increase in cortical microRNA biogenesis. *Mol Psychiatry* **15**, 1176-1189.
48. Santarelli, D. M., Beveridge, N. J., Tooney, P. A. & Cairns, M. J. (2011). Upregulation of dicer and microRNA expression in the dorsolateral prefrontal cortex Brodmann area 46 in schizophrenia. *Biol Psychiatry* **69**, 180-187.
49. Zhang, X., Cairns, M., Rose, B., O'Brien, C., Shannon, K., et al. (2009). Alterations in miRNA processing and expression in pleomorphic adenomas of the salivary gland. *Int J Cancer* **124**, 2855-2863.
50. Chiosea, S., Jelezcova, E., Chandran, U., Acquafondata, M., McHale, T., et al. (2006). Up-regulation of dicer, a component of the MicroRNA machinery, in prostate adenocarcinoma. *Am J Pathol* **169**, 1812-1820.
51. Chiosea, S., Jelezcova, E., Chandran, U., Luo, J., Mantha, G., et al. (2007). Overexpression of Dicer in precursor lesions of lung adenocarcinoma. *Cancer Res* **67**, 2345-2350.
52. Ambs, S., Prueitt, R. L., Yi, M., Hudson, R. S., Howe, T. M., et al. (2008). Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. *Cancer Res* **68**, 6162-6170.
53. Hill, D. A., Ivanovich, J., Priest, J. R., Gurnett, C. A., Dehner, L. P., et al. (2009). DICER1 mutations in familial pleuropulmonary blastoma. *Science* **325**, 965.

54. Karube, Y., Tanaka, H., Osada, H., Tomida, S., Tatematsu, Y., et al. (2005). Reduced expression of Dicer associated with poor prognosis in lung cancer patients. *Cancer Sci* **96**, 111-115.
55. Jakymiw, A., Patel, R. S., Deming, N., Bhattacharyya, I., Shah, P., et al. (2010). Overexpression of dicer as a result of reduced let-7 MicroRNA levels contributes to increased cell proliferation of oral cancer cells. *Genes Chromosomes Cancer* **49**, 549-559.
56. Blenkiron, C., Goldstein, L. D., Thorne, N. P., Spiteri, I., Chin, S. F., et al. (2007). MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. *Genome Biol* **8**, R214.
57. Cheng, C., Fu, X., Alves, P. & Gerstein, M. (2009). mRNA expression profiles show differential regulatory effects of microRNAs between estrogen receptor-positive and estrogen receptor-negative breast cancer. *Genome Biol* **10**, R90.
58. Faggad, A., Budczies, J., Tchernitsa, O., Darb-Esfahani, S., Sehouli, J., et al. (2010). Prognostic significance of Dicer expression in ovarian cancer-link to global microRNA changes and oestrogen receptor expression. *J Pathol* **220**, 382-391.
59. Kaneko, H., Dridi, S., Tarallo, V., Gelfand, B. D., Fowler, B. J., et al. (2011). DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. *Nature* **471**, 325-330.
60. Adams, B. D., Claffey, K. P. & White, B. A. (2009). Argonaute-2 expression is regulated by epidermal growth factor receptor and mitogen-activated protein kinase signaling and correlates with a transformed phenotype in breast cancer cells. *Endocrinology* **150**, 14-23.
61. Schutz, S. & Sarnow, P. (2006). Interaction of viruses with the mammalian RNA interference pathway. *Virology* **344**, 151-157.
62. Umbach, J. L. & Cullen, B. R. (2009). The role of RNAi and microRNAs in animal virus replication and antiviral immunity. *Genes Dev* **23**, 1151-1164.
63. Bennasser, Y. & Jeang, K. T. (2006). HIV-1 Tat interaction with Dicer: requirement for RNA. *Retrovirology* **3**, 95.
64. Bennasser, Y., Le, S. Y., Benkirane, M. & Jeang, K. T. (2005). Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. *Immunity* **22**, 607-619.
65. Coley, W., Van Duyne, R., Carpio, L., Guendel, I., Kehn-Hall, K., et al. (2010). Absence of DICER in monocytes and its regulation by HIV-1. *J Biol Chem* **285**, 31930-31943.
66. Bennasser, Y., Yeung, M. L. & Jeang, K. T. (2006). HIV-1 TAR RNA subverts RNA interference in transfected cells through sequestration of TAR RNA-binding protein, TRBP. *J Biol Chem* **281**, 27674-27678.
67. Andersson, M. G., Haasnoot, P. C., Xu, N., Berenjian, S., Berkhout, B., et al. (2005). Suppression of RNA interference by adenovirus virus-associated RNA. *J Virol* **79**, 9556-9565.
68. Aparicio, O., Carnero, E., Abad, X., Razquin, N., Guruceaga, E., et al. (2010). Adenovirus VA RNA-derived miRNAs target cellular genes involved in cell growth, gene expression and DNA repair. *Nucleic Acids Res* **38**, 750-763.
69. Aparicio, O., Razquin, N., Zaratiegui, M., Narvaiza, I. & Fortes, P. (2006). Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production. *J Virol* **80**, 1376-1384.
70. Bennasser, Y., Chable-Bessia, C., Triboulet, R., Gibbings, D., Gwizdek, C., et al. (2010). Competition for XPO5 binding between Dicer mRNA, pre-miRNA and viral RNA regulates human Dicer levels. *Nat Struct Mol Biol* **18**, 323-327.
71. de Vries, W., Haasnoot, J., van der Velden, J., van Montfort, T., Zorgdrager, F., et al. (2008). Increased virus replication in mammalian cells by blocking intracellular innate defense responses. *Gene Ther* **15**, 545-552.
72. Boudreau, R. L., Martins, I. & Davidson, B. L. (2009). Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo. *Mol Ther* **17**, 169-175.
73. Castanotto, D., Sakurai, K., Lingeman, R., Li, H., Shively, L., et al. (2007). Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. *Nucleic Acids Res* **35**, 5154-5164.
74. Giering, J. C., Grimm, D., Storm, T. A. & Kay, M. A. (2008). Expression of shRNA from a tissue-specific pol II promoter is an effective and safe RNAi therapeutic. *Mol Ther* **16**, 1630-1636.
75. Ehlert, E. M., Eggers, R., Niclou, S. P. & Verhaagen, J. (2010). Cellular toxicity following application of adeno-associated viral vector-mediated RNA interference in the nervous system. *BMC Neurosci* **11**, 20.
76. Martin, J. N., Wolken, N., Brown, T., Dauer, W. T., Ehrlich, M. E., et al. (2011). Lethal toxicity caused by expression of shRNA in the mouse striatum: implications for therapeutic design. Gene Ther Epub ahead of print doi: 10.1038/gt.2011.10
77. Pan, Q., de Ruiter, P. E., von Eije, K. J., Smits, R., Kwekkeboom, J., et al. (2011). Disturbance of the microRNA pathway by commonly used lentiviral shRNA libraries limits the application for screening host factors involved in hepatitis C virus infection. *FEBS Lett* **585**, 1025-1030.
78. Khan, A. A., Betel, D., Miller, M. L., Sander, C., Leslie, C. S., et al. (2009). Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs. *Nat Biotechnol* **27**, 549-555.
79. Beer, S., Bellovin, D. I., Lee, J. S., Komatsubara, K., Wang, L. S., et al. (2010). Low-level shRNA cytotoxicity can contribute to MYC-induced hepatocellular carcinoma in adult mice. *Mol Ther* **18**, 161-170.
80. Diederichs, S., Jung, S., Rothenberg, S. M., Smolen, G. A., Mlody, B. G., et al. (2008). Coexpression of Argonaute-2 enhances RNA interference toward perfect match binding sites. *Proc Natl Acad Sci U S A* **105**, 9284-9289.
81. Grimm, D., Wang, L., Lee, J. S., Schurmann, N., Gu, S., et al. (2010). Argonaute proteins are key determinants of RNAi efficacy, toxicity, and persistence in the adult mouse liver. *J Clin Invest* **120**, 3106-3119.
82. Boudreau, R. L., Monteys, A. M. & Davidson, B. L. (2008). Minimizing variables among hairpin-based RNAi vectors reveals the potency of shRNAs. *RNA* **14**, 1834-1844.
83. McBride, J. L., Boudreau, R. L., Harper, S. Q., Staber, P. D., Monteys, A. M., et al. (2008). Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc Natl Acad Sci U S A* **105**, 5868-5873.

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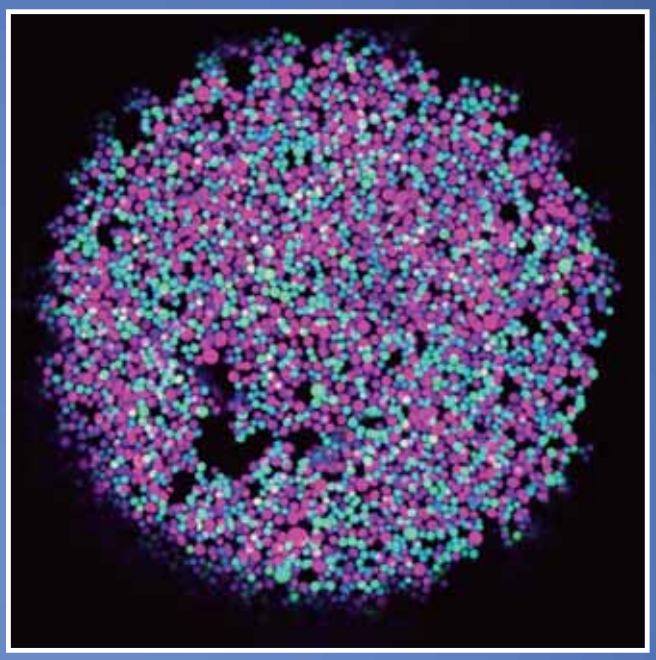

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The role of the co-chaperone BAG3 in selective macroautophagy: Implications for aging and disease

The proteins of the cell are in a constant turnover. They are synthesized, properly folded, targeted to specific intracellular sites where needed, and have a certain half-life before they are degraded. This physiological life cycle of proteins is described as protein homeostasis and is crucial for function and survival of any particular cell, ranging from highly proliferating to differentiated cells. Throughout their lifetime, cells face permanently changing and under certain circumstances rather unfriendly conditions that challenge also the protein turnover system. As consequence, especially the protein degradation activity is under pressure and cells invest quite some energy and effort to provide efficient protein degradation. In fact, cells have basically two separate protein degradation systems that differ, for instance in their mode of action, degradation efficacy, and substrate specificity. These two systems are the ubiquitin-proteasome system (UPS) and autophagy (here referred to also as macroautophagy) (Nandi et al., 2006; Mizushima, 2007) (Figure 1). While the proteasome system is rather well understood and defined, the macroautophagy pathway consists of a variety of separate degradation routes, involving a set of chaperones, co-chaperones, and adaptor proteins. In particular the role of co-chaperones in macroautophagy has recently gained a lot of attention.

Increasing demand for protein degradation during aging and disease: Enhanced protein aggregation is a prominent hallmark of aged cells and is associated and partially linked to the onset and progression of neurodegenerative disorders (Rautou et al., 2010; Dillin and Cohen, 2011). In age-associated neurodegenerative disorders, including Huntington Disease, Parkinson Disease, and amyotrophic lateral sclerosis (ALS), protein aggregates are found. Moreover, aggregated proteins as pathological hallmark are detected also in different non-neuronal diseases. For instance, protein deposits are found also in various liver pathologies, known there as Mallory-Denk bodies. Obviously, under unfavorable conditions as they occur during aging and in disease, cells are no longer able

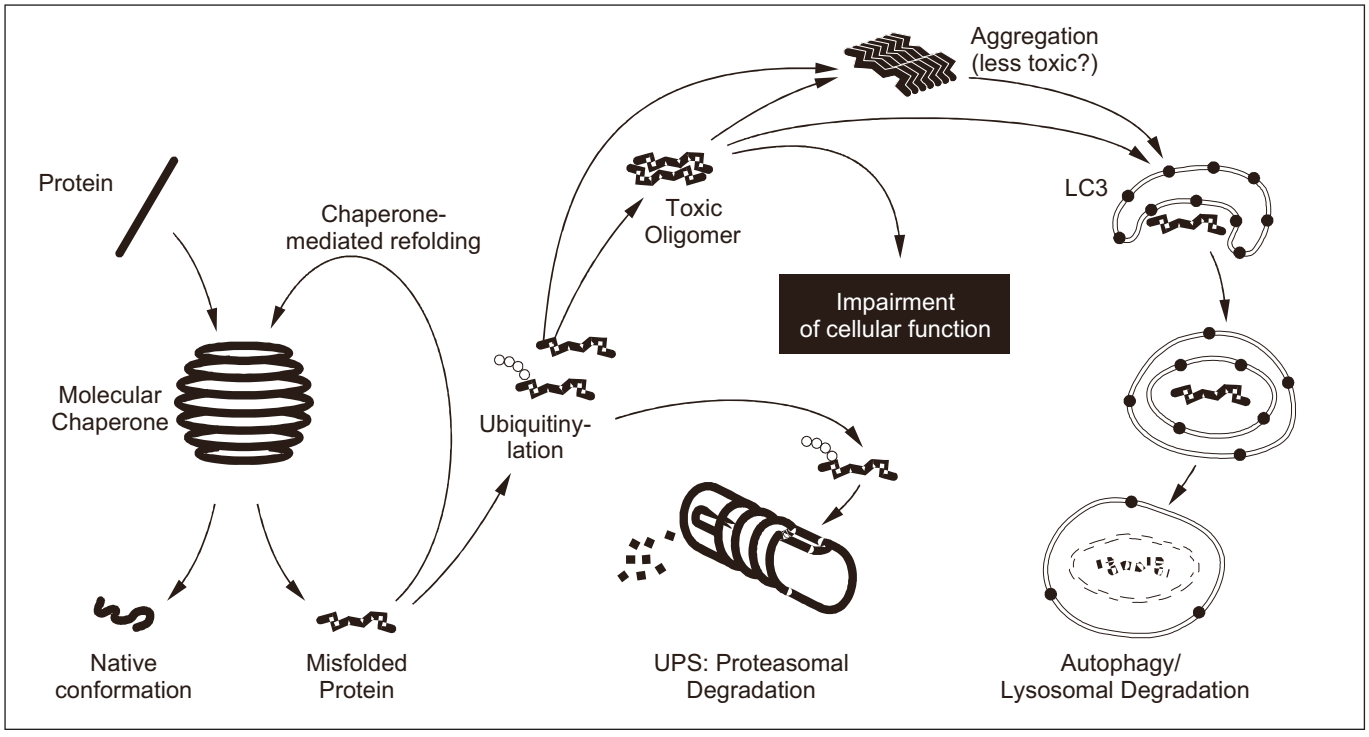

Fig. 1.: The two principal pathways of protein degradation: ubiquitin-proteasome system and (macro)autophagy.

to fully degrade accumulating proteins and deposit this protein junk inside the cell. During aging oxidative modifications of all cellular biomolecules are observed (Stadtman, 2006). One view is that the intracellular deposition of protein aggregates is a consequence of a wear-and-tear process driven by, for instance, oxidations as it can be seen in aged cells as deposits of lipufuscin. During disease also genetic and biochemical changes (e.g. mutations, folding errors) may lead to the increased propensity of proteins to aggregate. The HSP70 (heat shock protein 70) system of chaperones and co-chaperones appears to be involved in protein degradation, besides being responsible for folding and refolding of proteins (Kampinga and Craig, 2010; Young, 2010). In the neurodegenerative disease ALS, mutated forms of the antioxidant enzyme superoxide dismutase 1 (SOD1) have a tendency to aggregate. So far it is not dissolved whether the aggregation of mutated SOD1 is indeed the cause of neurotoxicity and, therefore, disease or a downstream event. In fact, the neurotoxic effects have been described also to be partially independent of the extent of aggregation (Witan et al., 2008; Witan et al., 2009). Nevertheless, deposition of protein junk might cause secondary effects and is at least an additional challenge for the physiology of the cell rendering it potentially more vulnerable. A clear visible sign of age-associated protein deposition is the occurrence of lipofuscin, a not-well defined collection of crosslinked proteins and lipids that can be seen in epidermal cells of the skin but is also found in other tissues (e.g. liver, brain). Interestingly, activation of the autophagy pathways with the drug rapamycin leads to enhanced clearance of aggregates of mutated huntingtin, the hallmark of Huntington Disease (Rose et al., 2010). The induction of autophagy pathways with small molecules is currently discussed as one novel therapeutic option for neurodegenerative disorders accompanied by intracellular protein aggregation and deposition (Fleming et al., 2011). But since different routes of intracellular autophagy are known and enhanced autophagy turnover has been linked to cancer development and progression (Levine and Kroemer, 2008) a specific activation of selective autophagy pathways in contrast to the general induction of various autophagy pathways at once should be considered.

The different routes of autophagy in a nutshell: In general, autophagy is a ubiquitous and evolutionarily conserved process in eukaryotes that degrades cytosolic components by the lysosome (Johansen and Lamark, 2011). So far three different types of autophagy have been described in detail: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Mizushima, 2007; Arias and Cuervo, 2011). The autophagy process itself also includes a degradation pathway called mitophagy that selectively eliminates organelles (mitochondria) to regulate their number and maintain organelle homeostasis (Youle and Narendra, 2011). Autophagy (in the following now referred to as macroautophagy) was initially thought to be an unspecific bulk and robust degradation process. Based on the identification of various molecular players and modifiers today it is clear that macroautophagy pathways exist that remove substrates in a highly selective manner (Johansen and Lamark, 2011). In fact, macroautophagy is not a crude digestion process but follows a sophisticated multi-step mechanism and interplay of membranes and proteins. Cytosolic material, for instance misfolded and aggregated proteins, is sequestered in a double-layered membrane structure, the so-called autophagosome, and delivered to the degradation compartment, the lysosome. While macroautophagy has been considered for quite some time as process lacking substrate specificity, microautophagy involves direct sequestration of cytosolic components by invagination of the lysosomal membrane. In contrast, CMA displays a high selectivity that removes only a distinct subset of proteins carrying a pentapeptide lysosome-targeting motif (KFERQ). Such substrates are directly translocated into the lysosome after binding to the lysosomal receptor LAMP2A. For the translocation into the lysosome substrates are then unfolded by a chaperone complex containing HSC70 and the co-chaperones BAG1, Hip, Hop, and HSP40/DNAJB1 (Arias and Cuervo, 2011). Focusing on these autophagy types, in particular macroautophagy has recently attracted a lot of attention because it becomes increasingly clear that, (1) in addition to the UPS, macroautophagy plays an essential role in maintaining protein homeostasis, (2) also macroautophagy can display a selectivity in substrate clearance, and (3) an interplay of chaperones and co-chaperones mediates the selection of degradation-prone proteins (Dice, 2007; Carra et al., 2008a; Johansen and Lamark, 2011; Behl, 2011). Indeed, a huge step forward was the discovery of molecular adaptors of autophagy, the proteins SQSTM1/p62 (sequestosome-1/p62) and NBR1 (neighbor of Brc1). These adaptors play a central role as cargo receptors and functional interfaces for the degradation of ubiquitinated protein substrates. Moreover, the finding of the direct interaction between SQSTM1/p62 and NBR1 with LC3, a protein of the autophagosome, has shed new light on the macroautophagy route (Johansen and Lamark, 2011).

BAG1 and BAG3 are reciprocally expressed and display different functions: While investigating basic biochemical differences of young and old cells, we found the expression of two members of the BAG (Bcl-2-associated athanogene) family, BAG1 and BAG3, reciprocally regulated during cellular aging as well as under acute stress (e.g. oxidative stress or proteasomal inhibition). This reciprocal expression of these two co-chaperones is called "BAG1/BAG3-switch" (Gamerdinger et al., 2009; Behl, 2011). Under normal physiological conditions protein quality control (PQC) is mainly mediated by the activity of HSP70 towards misfolded proteins. In this process BAG1 obviously plays a major role, since BAG1 while interacting with HSP70 is essen-

tial for substrate transfer and proteasomal degradation of poly-ubiquitinated (polyUb)-proteins (Gamerdinger et al., 2009). When the cellular environment changes and becomes unfriendly, representing pathophysiological conditions as during aging and acute stress, an accumulation of misfolded and aggregated proteins can be observed. Moreover, the proteasomal degradation efficacy is decreased during aging and under non-physiological conditions. Here, BAG3 expression is increased and, most importantly, as consequence of the accumulation of degradation-prone proteins, the BAG3-mediated selective macroautophagy is turned on. This "on-demand macroautophagy" is potentially of vital importance for cell survival and is an adaptive response.

HSP70 provides substrate specificity: An essential prerequisite of BAG3-mediated macroautophagy is the cooperation with HSP70. In fact, substrate specificity for misfolded proteins is based on the particular function of HSP70. The HSP70 chaperone system is complex, consisting of the main chaperone HSP70 and a larger group of co-chaperones and co-regulators (Hartl and Hayer-Hartl, 2002; Lanneau et al., 2010; Young, 2010) and represents the intracellular detection system for unfolded and misfolded substrates. It specifically detects such substrates and transfers them to the intracellular protein degradation machinery. This substrate identification occurs via the activity of molecular chaperones that have a high binding affinity for solvent-exposed, unstructured and hydrophobic regions of non-native proteins. Under unfavourable or protein-denaturing conditions including heat stress many molecular chaperones are up-regulated. Therefore these proteins are referred to as stress proteins or heat-shock proteins (HSP) (Hartl and Hayer-Hartl, 2002; Lanneau et al., 2010; Young, 2010). When HSP70 binds to substrates unstructured polypeptides can fold (or refold) into the native and functional three-dimensional structure. But, when this native state cannot be reached, like in the case of disease-related mutated proteins (e.g. polyQ-expanded huntingtin, mutant SOD1, α-synuclein), molecular chaperones change their function and can promote subsequent substrate degradation (Hartl and Hayer-Hartl, 2002; Chang et al., 2007; Tang et al., 2007). The binding of substrates to HSP70 and their subsequent release is regulated by ATP in an ATP-consuming cycle. When ATP is bound to HSP70 binding of substrate occurs rather rapid. In this state, the hydrolysis of bound ATP to ADP stabilizes the direct interaction of substrate with HSP70. The following release of bound ADP from HSP70 and the subsequent rebinding of a novel molecule ATP induce the dissociation of the chaperone-substrate complex and the release of the substrate. The activity of HSP70 in substrate folding and refolding as well as the degradation activity is regulated by co-chaperones that influence the ATP-consuming cycle (e.g. HSP40, HIP). Moreover, other HSP70-binding co-factors play a role including the ubiquitin ligases CHIP and parkin (Young, 2010).

BAG3-mediated selective macroautophagy: Proteins of the BAG protein family which includes in total six members in humans (Takayama and Reed, 2001) are key mediators of HSP70-assisted protein degradation pathways. The evolutionary conserved BAG domain allows these proteins to interact directly with HSP70 and to modify and regulate HSP70 function. BAG proteins directly affect the ATP-consuming cycle and regulate the ATP/ADP exchange on HSP70. Thereby, they trigger the release of a chaperone-bound substrate from HSP70 (Sondermann et al., 2001). Subsequently, BAG proteins may couple the released sub-

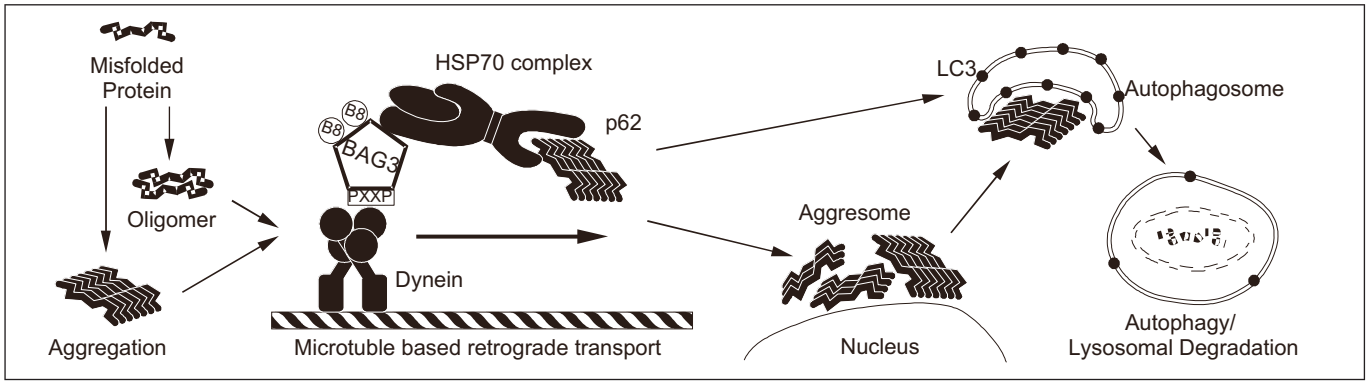

Fig. 2.: Putative mechanism of action of the BAG3-HSPB8-HSP70 chaperone complex: Protein misfolding, either due to genetic mutation or to external stress (e.g. heat shock, oxidative stress), leads to protein instability and aggregation. Misfolded aggregation-prone proteins are recognized and bound by the molecular chaperones HSPB8 and HSP70, which in complex with the co-chaperone BAG3 target them to macroautophagy for degradation. In particular, through its PxxP region, BAG3 associates with the dynein motor protein, which allows the retrograde transport of the misfolded proteins to the microtubule organizing center. Here, aggregated proteins are assembled together to form the aggresome, a structure that acts as staging ground for the disposal of protein aggregates by autophagy. Interaction of BAG3 with the LC3-binding protein p62 may further ensure the specific targeting of the misfolded proteins to the autophagic vacuoles for degradation.

strates to specific downstream cellular processes, such as the degradation pathways. For instance, BAG1 couples HSP70 to the proteasomal degradation pathway and is mediating the transfer of substrate to the proteasome. BAG3 on the other hand has been recently shown to specifically control HSP70-assisted protein degradation by the main alternative protein degradation route, the macroautophagy (Carra et al., 2008b; Gamerdinger et al., 2009). Although BAG3 while directly interacting with HSP70 is in the core of this novel identified selective macroautophagy pathway, additional players, such as other co-chaperones are necessary. The recruitment of the BAG3-mediated macroautophagy pathway involves polyUb-substrates, HSP70, HSPB8, SQSTM1/p62 and LC3 (Carra et al., 2008a; Gamerdinger et al., 2009). In fact, the multi-chaperone protein complex BAG3-HSPB8-HSP70 was found to control selective degradation of substrates such as polyUb-proteins and disease proteins including polyQ-huntingtin and superoxide dismutase-1 mutants linked to ALS (Crippa et al., 2010; Gamerdinger et al., 2011). Interestingly, more recently, we found that the substrates for this BAG3-driven macroautophagy pathway do not have to be necessarily ubiquitinated, since non ubiquitinated proteins can also be clients of this particular BAG3-mediated macroautophagy pathway (Gamerdinger et al., 2011). Due to its domain structure BAG3 can interact with many protein partners linking it besides to autophagy also to a variety of other key cellular pathways, including apoptosis and proliferation (McCollum et al., 2010).

BAG3 controls aggresome targeting and interacts with dynein: It has been known for quite some time that damaged, misfolded and aggregated proteins (as autophagy substrates) are not randomly distributed throughout the cell but are found at specific intracellular sites. Indeed, selective macroautophagy strongly requires the concentration and separation of degradation substrates away from other cytosolic components. The sequestration of degradation substrates in a special perinuclear compartment, called the aggresome, is realized by a retrograde transport of the substrates along microtubules via the cytoplasmic dynein motor complex (Kopito, 2000). Interestingly, we have found that the co-chaperone BAG3 directly associates with the microtubule motor dynein and mediates the selective transport of misfolded proteins to the aggresome. The binding of BAG3 to dynein occurs through a PxxP domain at BAG3 (Gamerdinger et al., 2011) (Figure 2). Interestingly, the inhibition of dynein-mediated transport and, consequently, the prevention of aggresome formation, lead to an inefficient degradation of aggregation-prone proteins by macroautophagy and is associated with the progression of various disorders, including ALS (Ravikumar et al., 2005). BAG3 is directly colocalized with aggresome markers and an enhanced BAG3 expression increases the aggresome formation, whereas its knock down by RNAi reduces the number of perinuclear aggresomes (Gamerdinger et al., 2011). Therefore, the following model is proposed: BAG3 functions as an ATP/ADP-exchange factor and, thus, directly stimulates the release and transfer of substrate from HSP70 that provides substrate selectivity to the dynein motor complex. Subsequently, the degradation substrates are transported along the dynein/microtubules complex to a perinuclear site. Therefore, BAG3 directly promotes the shuttle of misfolded proteins to the aggresome and their concentration at a perinuclear site. In a direct interaction with SQSTM1/p62 and later on with LC3 the transfer into the macroautophagy pathway is possible. A link of aggresomes to the macroautophagy pathway has been described (Johnston et al., 1998; Webb et al., 2004). Taken together, BAG3 in complex with HSPB8 and HSP70 serves a key function in the sequestration of degradation substrates and their transport into aggresomes and may serve also as direct link to SQSTM1/p62 that binds to ubiquitin as well as to LC3 to switch on macroautophagy (Figure 2). This process is highly relevant for disorders that are associated with protein aggregrates. Disease proteins, such as polyQ-huntingtin or mutated SOD1, cannot be handled by the proteasome and they hence accumulate. Consequently, the BAG3-HSP8-HSP70 multi-chaperone complex promotes targeting of these protein aggregates to the aggresome and their subsequent degradation. As already mentioned, enhanced protein aggregation is a hallmark of aging and various neurodegenerative diseases. It is well known that targeting of substrates to the aggresome is achieved by the ubiquitination of degradation-prone proteins that are recognized by ubiquitin binding proteins, such as the deacetylase HDAC6, binding to dynein (Kawaguchi et al., 2003; Yao, 2010; Li et al., 2011). Because many misfolded proteins are not ubiquitinated, selective loading of cargo onto dynein must also occur independently of ubiquitination. Here, the co-chaperone BAG3 might play the key role.

In summary, the described novel pathway of aggresome targeting and selective macroautophagy mediated by BAG3 in concert with other co-chaperones is an important example for the involvement of chaperone molecules in autophagy and for a fine-tuned mechanism of macroautophagy. Selective macroautophagy mediated by specific co-chaperones could be considered as novel target for prevention and therapy of age-associated neurodegeneration in the future.

Taken together: Maintenance of protein homeostasis, correct protein folding, refolding and clearance is of central importance for the function and survival of every cell. Here, the degradation of proteins is of particular importance, especially during aging and certain degenerative disorders when the protein load is increased. During cellular

aging as well as under acute stress there is a reciprocal change in expression of two members of the BAG family, BAG1 and BAG3. While BAG1 serves an important function during the degradation of ubiquitinated proteins via the proteasome, BAG3 is the mediator of a novel macroautophagy pathway. This BAG3-mediated macroautophagy is based on the specificity of HSP70 for misfolded proteins and involves also other protein partners, such as HSPB8, SQSTM1/p62 and the autophagosome protein LC3. BAG3 directly mediates the targeting and transport of degradation-prone substrates into aggresomes via the microtubule-motor dynein and works also independent of substrate ubiquitination.

References

Arias E, Cuervo AM (2011) Chaperone-mediated autophagy in protein quality control. *Curr Opin Cell Biol* **23**:184-9.

Behl C (2011) BAG3 and friends: Co-chaperones in selective autophagy during aging and disease. *Autophagy*, **7**:1-4.

Carra S, Seguin SJ, Landry J (2008a) HspB8 and Bag3: a new chaperone complex targeting misfolded proteins to macroautophagy. *Autophagy* **4**:237-239.

Carra S, Seguin SJ, Lambert H, Landry J (2008b) HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. *J Biol Chem* **283**:1437-1444.

Chang HC, Tang YC, Hayer-Hartl M, Hartl FU (2007) SnapShot: molecular chaperones, Part I. *Cell* **128**:212.

Crippa V, Sau D, Rusmini P, Boncoraglio A, Onesto E, Bolzoni E, Galbiati M, Fontana E, Marino M, Carra S, Bendotti C, De Biasi S, Poletti A (2010) The small heat shock protein B8 (HspB8) promotes autophagic removal of misfolded proteins involved in amyotrophic lateral sclerosis (ALS). *Hum Mol Genet* **19**:3440-3456.

Dice JF (2007) Chaperone-mediated autophagy. *Autophagy* **3**:295-299.

Dillin A, Cohen E (2011) Ageing and protein aggregation-mediated disorders: from invertebrates to mammals. *Philos Trans R Soc Lond B Biol Sci* **366**:94-98.

Fleming A, Noda T, Yoshimori T, Rubinsztein DC (2011) Chemical modulators of autophagy as biological probes and potential therapeutics. *Nat Chem Biol* **7**:9-17.

Gamerdinger M, Kaya AM, Wolfrum U, Clement AM, Behl C (2011) BAG3 mediates chaperone-based aggresome-targeting and selective autophagy of misfolded proteins. *EMBO Rep* **12**:149-156.

Gamerdinger M, Hajieva P, Kaya AM, Wolfrum U, Hartl FU, Behl C (2009) Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. *EMBO J* **28**:889-901.

Hartl FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**:1852-1858.

Johansen T, Lamark T (2011) Selective autophagy mediated by autophagic adapter proteins. *Autophagy* **7**:279-296.

Johnston JA, Ward CL, Kopito RR (1998) Aggresomes: a cellular response to misfolded proteins. *J Cell Biol* **143**:1883-1898.

Kampinga HH, Craig EA (2010) The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nat Rev Mol Cell Biol* **11**:579-592.

Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP (2003) The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell* **115**:727-738.

Kopito RR (2000) Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol* **10**:524-530.

Lanneau D, Wettstein G, Bonniaud P, Garrido C (2010) Heat shock proteins: cell protection through protein triage. *ScientificWorldJournal* **10**:1543-1552.

Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. *Cell* **132**:27-42.

Li G, Jiang H, Chang M, Xie H, Hu L (2011) HDAC6 alpha-tubulin deacetylase: A potential therapeutic target in neurodegenerative diseases. *J Neurol Sci* **304**:1-8.

McCollum AK, Casagrande G, Kohn EC (2010) Caught in the middle: the role of Bag3 in disease. *Biochem J* **425**:e1-3.

Mizushima N (2007) Collaboration of proteolytic systems. *Autophagy* **3**:179-180.

Nandi D, Tahiliani P, Kumar A, Chandu D (2006) The ubiquitin-proteasome system. *J Biosci* **31**:137-155.

Rautou PE, Mansouri A, Lebrec D, Durand F, Valla D, Moreau R (2010) Autophagy in liver diseases. *J Hepatol* **53**:1123-1134.

Ravikumar B, Acevedo-Arozena A, Imarisio S, Berger Z, Vacher C, O'Kane CJ, Brown SD, Rubinsztein DC (2005) Dynein mutations impair autophagic clearance of aggregate-prone proteins. *Nat Genet* **37**:771-776.

Rose C, Menzies FM, Renna M, Acevedo-Arozena A, Corrochano S, Sadiq O, Brown SD, Rubinsztein DC (2010) Rilmenidine attenuates toxicity of polyglutamine expansions in a mouse model of Huntington's disease. *Hum Mol Genet* **19**:2144-2153.

Sondermann H, Scheufler C, Schneider C, Hohfeld J, Hartl FU, Moarefi I (2001) Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. *Science* **291**:1553-1557.

Stadtman ER (2006) Protein oxidation and aging. *Free Radic Res* **40**:1250-1258.

Takayama S, Reed JC (2001) Molecular chaperone targeting and regulation by BAG family proteins. *Nat Cell Biol* **3**:E237-241.

Tang YC, Chang HC, Hayer-Hartl M, Hartl FU (2007) SnapShot: molecular chaperones, Part II. *Cell* **128**:412.

Webb JL, Ravikumar B, Rubinsztein DC (2004) Microtubule disruption inhibits autophagosome-lysosome fusion: implications for studying the roles of aggresomes in polyglutamine diseases. *Int J Biochem Cell Biol* **36**:2541-2550.

Witan H, Kern A, Koziollek-Drechsler I, Wade R, Behl C, Clement AM (2008) Heterodimer formation of wild-type and amyotrophic lateral sclerosis-causing mutant Cu/Zn-superoxide dismutase induces toxicity independent of protein aggregation. *Hum Mol Genet* **17**:1373-1385.

Witan H, Gorlovoy P, Kaya AM, Koziollek-Drechsler I, Neumann H, Behl C, Clement AM (2009) Wild-type Cu/Zn superoxide dismutase (SOD1) does not facilitate, but impedes the formation of protein aggregates of amyotrophic lateral sclerosis causing mutant SOD1. *Neurobiol Dis* **36**:331-342.

Yao TP (2010) The role of ubiquitin in autophagy-dependent protein aggregate processing. *Genes Cancer* **1**:779-786.

Youle RJ, Narendra DP (2011) Mechanisms of mitophagy. *Nat Rev Mol Cell Biol* **12**:9-14.

Young JC (2010) Mechanisms of the Hsp70 chaperone system. *Biochem Cell Biol* **88**:291-300.

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Since 2002	Full Professor for Pathobiochemistry, Chair of the Department of Pathobiochemistry, Director of the Institute for Pathobiochemistry, Johannes Gutenberg University Mainz
1999	Habilitation, Medical Faculty, Ludwig Maximilians University, Munich
1997 - 2002	Head of an Independent Max Planck Society Research Group, Max Planck Institute of Psychiatry (Director: Prof. Dr. Dr. F. Holsboer), Munich
1994 - 1996	Research Scientist, Head of the Steroidpharmacology Group, Max-Planck Institute of Psychiatry, Munich
1992 - 1994	Postdoctoral Researcher, Salk Institute for Biological Studies (Prof. Dr. D. Schubert), San Diego (USA)
1991 - 1992	Postdoctoral Fellow, Department of Neurology, Julius Maximilians University, Würzburg
1991	Dr. rer. nat., Neurobiology, Department of Neurology and Institute for Genetics, Julius Maximilians University, Würzburg
1988	Dipl.-Biol., Julius Maximilians University, Würzburg

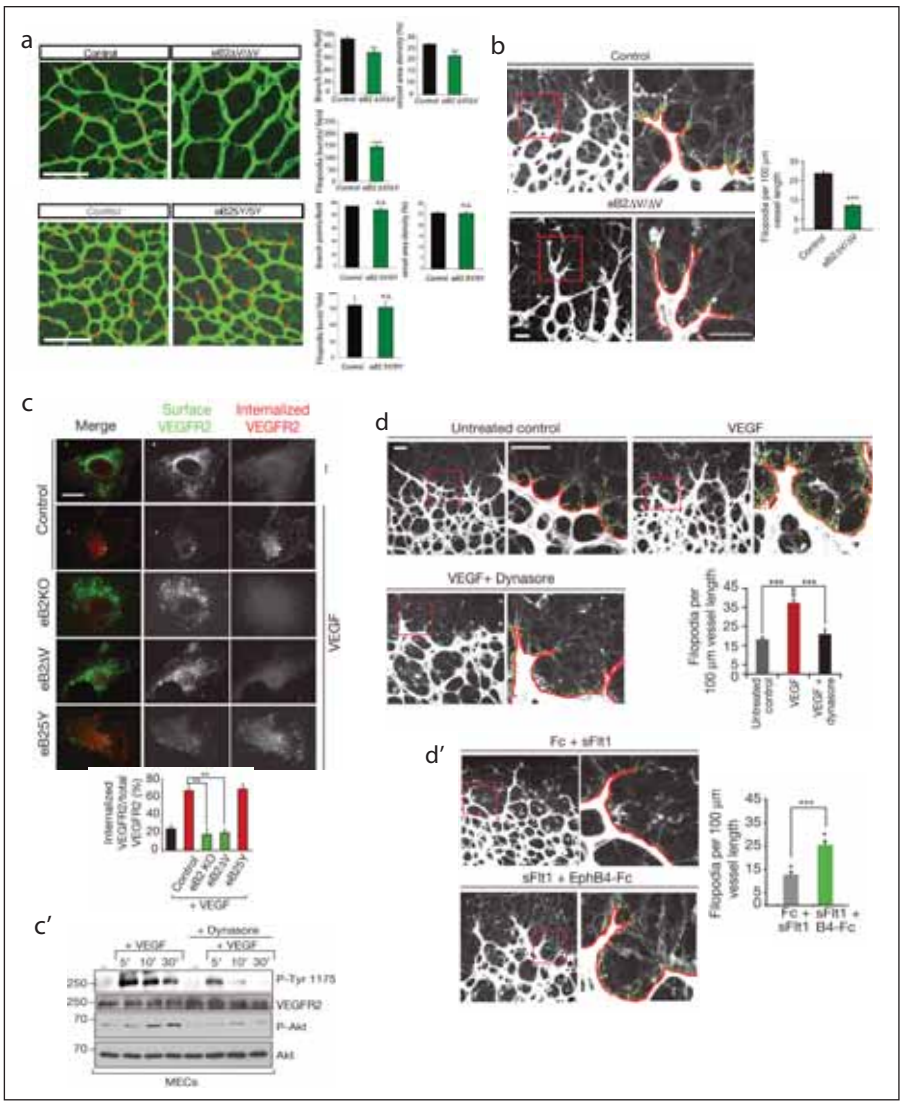
Werner Risau Prize 2011: Suphansa Sawamiphak

Controlling endothelial tip cell behavior: a team effort of EphrinB2 and VEGFR2

Wiring of the vascular system into a complex hierarchical network that is capable of effective delivery of oxygen and nutrients to different organs throughout the body is of utmost importance and requires the precisely coordinated series of guidance decisions. Clues to the complex nature of vessel guidance come from the anatomical similarities of the vascular and nervous network and moreover, their paralleled developmental patterns (Mukouyama et al., 2002). The task of axon navigation through a complex environment over considerable distances to reach the appropriate targets is carried out by a specialized structure at the leading tip of growing axons, the growth cones. The filopodia-riched structure and the dynamic nature of the growth cone hold striking similarities to the specialized cells at leading front of growing vessel network so called 'tip cells'. By continually extending and retracting filopodial processes, function as a sensor for guidance cues in the surrounding tissues and might also serve as physical clutches that stabilize cell-extracellular matrix contacts (Gerhardt et al., 2003; Lu et al., 2004), these tip cells represent a major driving force for forward movement of vascular sprouts toward correct routes. Extensive studies in these recent years have provided a solid proof of the pivotal role of these tip cells in angiogenic growth of blood vessel and pushed forward our understanding of the molecular players in control of tip cell specification (Benedito et al., 2009; Hellstrom et al., 2007; Siekmann and Lawson, 2007). On the contrary, our current knowledge of the molecular machineries that regulate guidance of tip cells is very limited.

In support to the structural and behavioral similarities, increasing evidences implicate that nerves and vessels may also share guidance molecules which transduce environmental cues to cytoskeletal changes required for cell migration (Adams and Eichmann; Carmeliet and Tessier-Lavigne, 2005). Bidirectional signaling from the Eph receptors and their transmembrane ligand ephrins represents one of the most important guidance cues involved in axon path finding (Bashaw and Klein; Egea and Klein, 2007). Interestingly, studies using mice with genetic mutation of a particular ligand EphrinB2 rendering it unable to transmit its intrinsic signaling have demonstrated that the ligand is indispensable for angiogenic remodeling of both blood and lymphatic vessels (Adams et al., 2001; Makinen et al., 2005). This led us to an attractive speculation that ephrinB2 might be involved in guided migration of growing vascular sprout. To address this hypothesis we analyzed the development of the vasculatures in ephrinB2 signaling mutant mice using postnatal retina model where vascular growth occurs exclusively by angiogenic sprouting in a highly reproducible pattern (Dorrell and Friedlander, 2006). We found that the lack of ephrinB2 PDZ target site (ephrinB2$\triangle$V), but not the disruption of its tyrosine-phosphorylation (ephrinB25Y), causes defective vascular development (Fig. 1a). Importantly, ephrinB2$^{\triangle V/\triangle V}$ mice exhibit a severe reduction of filopodial density at the sprouting front of expanding vascular network (Fig. 1b), implicated the importance of ephrinB2 PDZ-signaling in the formation and/or stabilization of the filopodial processes required for guided migration of endothelial tip cells. Interaction between the most prominent pro-angiogenic factor VEGF secreted from astrocytes and localized in the extracellular matrix within the close proximity to the astrocyte surface, and its receptor VEGFR2 located on the endothelial tip cell filopodia has been proposed to provide the initial establishment of astrocyte-endothelial contacts that mediate endothelial cell guidance along the pre-existing astrocytic scaffold during the vascularization of the retina (Gerhardt et al., 2003). This raised an appealing question whether the impairment of filopodial extensions in ephrinB2$^{\triangle V/\triangle V}$ mutant tip cells could be a result of misregulated VEGFR2 function indicating a molecular crosstalk between ephrinB2 reverse and VEGFR2 signaling.

Intracellular compartmentalization of signaling receptors has been supported by several compelling evidences to represent a critical mechanism that cells use to strictly regulate signal transduction temporally and spatially (Sorkin and von Zastrow, 2009). In classical belief, receptors are removed from the cell surface and en route directly to the proteolytic degradation only to dampen cellular response to extracellular signals. Indeed several receptors endocytosed and sorted into different endosomal compartments are now recognized to continue their signaling as in the case for EGF and NGF receptors (Howe et al., 2001; Sigismund et al., 2008). Moreover, the endocytic machinery has also been shown to play a key role during directional migration when polarized cellular response is critical



(Gould and Lippincott-Schwartz, 2009; Jekely et al., 2005; Palamidessi et al., 2008). Employing an immunofluorescent-based 'antibody feeding' assay we showed that ephrinB2 signaling through PDZ-binding domain is essential for the internalization of VEGFR2 from endothelial cell surface (Fig 1c). The next immediate question is if this function of ephrinB2 to mediate the internalization of VEGFR2 would be relevant for the receptor signaling activity. Upon ligand binding, VEGFR2 is dimerized and initiate trans autophosphorylation. Several tyrosine phosphorylation sites in the intracellular domain of the receptor serve as docking sites for the recruitment of various effector molecules to transduce receptor signaling (Olsson et al., 2006). In vivo analysis of VEGFR2 phosphorylation showed that activation of the receptor is largely compromised in ephrinB2$^{\triangle V/\triangle V}$ mutant mice. Moreover, using an inhibitor of dynamin-dependent endocytosis we found that, against earlier dogma, internalization of VEGFR2 is necessary to trigger sustained signaling downstream of the receptor over a prolonged period as demonstrated with its well-known mitogenic and migratory signaling effector Akt (Fig 1c').

The finding of the novel role of ephrinB2 to mediate internalization of VEGFR2 together

***Fig. 1.*: EphrinB2 mediates VEGFR2 endocytosis to control endothelial tip cell behavior. a**, PDZ-dependent signaling but not tyrosine phosphorylation of ephrinB2 is indipensable for vascular branching and sprouts formation. Retinal Vasculature of P7 mice were visualized by isolectin-B4. Decreased vessel density, branching, and tip cell sprouting (tip cells are indicated by red asterisks) in ephrinB2$^{\triangle V/\triangle V}$ mice (eB2 $\triangle$V/$\triangle$V) compared to wild type littermates (control) were clearly observed. By contrast, ephrinB2$^{5Y/5Y}$ (eB2 5Y/5Y) mice showed no significant difference from wild type control. Scale bars, 75 µm. **b**, Disruption of ephrinB2 PDZ-dependent signaling causes severe defect in endothelial tip cell filopodial extension. Right panel are closed up images of the vascular front areas as indicated by the red box in the left panel. Filopodial extensions (green dots) were quantified along the length of the vessel front (red line). Scale bars, 25 µm. **c**, EphrinB2 PDZ-signaling is necessary for VEGFR2 internalization. Endocytosis of VEGFR2 in primary endothelial cells was visualized by antibody feeding assay. Upon VEGF-stimulation VEGFR2 in ephrinB2-deficient (ephrinB2$^{lox/lox}$; Cre+, eB2 KO) and ephrinB2 PDZ signaling-deficient (ephrinB2$^{\triangle V/\triangle V}$, eB2$\triangle$V) cells largely remained on the cell surface, whereas ephrinB2 phosphotyrosine-signaling deficient (ephrinB2$^{5Y/5Y}$, eB25Y) cells showed similar level of internalized receptor as compared to EphrinB2$^{lox/lox}$ (control) cells. Scale bar, 25 µm. **c'**, Activation and prolongation of signaling downstream of VEGFR2 require receptor endocytosis. Mouse endothelial cells (MECs) were left untreated or pre-treated for 2 h with Dynasore and subsequently stimulated for the indicated times with VEGF. Activation of the receptor was assessed by phosphorylation on Tyr1175 in total cell lysates. VEGFR2 downstream signaling was assessed by level of Akt phosphorylation. Inhibition of endocytosis causes a drastic decrease of VEGFR2 phosphorylation and impairment of its signal transduction, affecting both intensity and duration of the signaling. **d**, EphrinB2 and VEGFR2 act in collaboration to regulate tip cell filopodial dynamics in an endocytic-dependent manner. Treatment with VEGF induced tip cell filopodial extensions in postnatal retinal explants. When endocytosis is disturbed in the presence of Dynasore, VEGF-stimulation failed to promote such migratory behavior. **d'**, Sequestration of endogenous VEGF with sFlt1 inhibited filopodial sprouting. Activation of ephrinB2 signaling with EphB4-Fc but not hFc was able to rescue number of filopodia to basal level. Vessels were stained with isolectin-B4. In right panels are high magnification pictures of the same vascular beds shown in left panels. Green dots indicate filopodia and red lines show the length of vascular front. Scale bars, 25 µm. Error bars represent SEM. Two-tailed t-test, * P< 0.05, ** P < 0.01, *** P < 0.001. Reproduced with permission from Sawamiphak et.al., 2010.

with the reported function of VEGFR2 in control of tip cell sprouting activity (Gerhardt et al., 2003) urge an appealing assumption that the regulation of filopodial extension by ephrinB2 might involve its capability to trigger VEGFR2 endocytosis and downstream signaling. To test this, we have developed a short-term culture system that allows us to study acute response of endothelial tip cell to extracellular substances. Employing retina explantation we showed that extension of filopodial structures in response to stimulation of VEGFR2 also requires functional endocytic process (Fig. 1d), further proven the functional relevance of receptor internalization for the proper signal transduction. To further verify the entwined relation between VEGFR2 and ephrinB2 signaling cascades and the dependency of the former on the latter to exert its function in tip cell, the ability of ephrinB2 to trigger activation of VEGFR2 signaling and consequently rescue the cellular outcome of inactivated VEGFR2 was examined. Treatment of explanted retinas with soluble VEGFR1 (Flt1), which can bind VEGFA with high affinity and thereby acts as a competitor to seclude the ligand produced endogenously in the retina from VEGFR2, resulted in a complete retraction of endothelial tip cell filopodia at the sprouting front of retinal vasculature. Importantly, activation of ephrinB2 signaling with preclustered EphB4-Fc fusion protein is able to diminish this inhibitory effect and rescue filopodial extension of the growing vessel (Fig. 1d'), underscore the credible role of ephrinB2 as a prime regulator of VEGFR2 internalization and activation of its downstream signaling to control the active sprouting activity of endothelial tip cells.

Tumors are long known to trigger angiogenic growth of blood vessels in host organs in order to attain oxygen and nutrients sufficient to supply rapid growth of tumor masses as well as to gain access to remote tissue by intravasation into the blood stream. To address the role of ephrinB2 signaling via PDZ-interaction on tumor progression we generated high-grade syngenic astrocytomas and injected the cells intracranially into ephrinB2$^{\Delta V/\Delta V}$ mice. Tumor growth in the mutant was severely reduced reaching less than 25% of control tumors grown in wild type littermates (Fig. 2a). The stunted tumor growth in the mutant was associated with striking reduction of tumor vascularization (Fig. 2b). Morphologically, the vasculature of control tumors was hyperdilated and torturous, reflecting the unusual high level of pro-angiogenic stimulants that shift the balance in the angiogenesis program in favor of vessel expansion and therefore hinder maturation processes. Vascular sprouts and filopodial extensions were readily detectable on these tumor vessels. By contrast, the vasculature of tumors grown in ephrinB2$^{\Delta V/\Delta V}$ mice was less torturous and resembled the normal brain vessels found under physiological conditions. Importantly, similar to the angiogenic sprouting defects observed in ephrinB2$^{\Delta V/\Delta V}$ mutant retinas, the filopodia-rich newly formed vascular sprouts were barely detectable in these tumors (Fig. 2b). This finding points out the important role of ephrinB2 PDZ-dependent signaling in tumor-induced angiogenic sprouting and tumor growth.

Taken together, our work reveals the novel molecular machinery in control of vascular sprouting whereby ephrinB2, through PDZ-interactions, regulates endocytosis and activation of VEGFR2 signaling required for

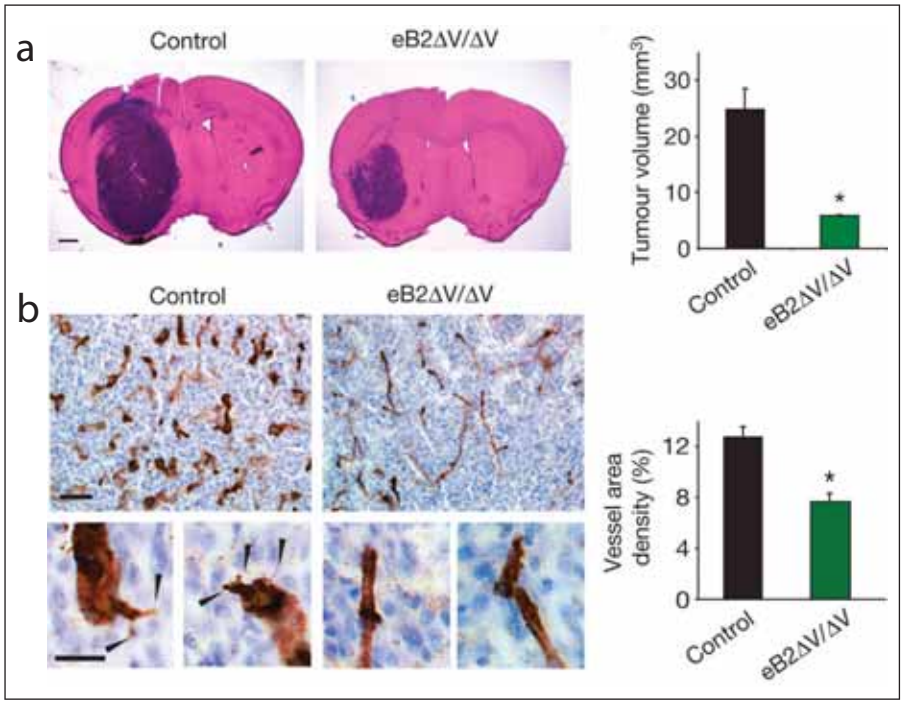


Fig.2 Blockade of ephrinB2 PDZ-signaling decreases brain tumor growth and reduces angiogenic sprouting of tumor vasculature. a, Intracranial tumor growth in ephrinB2$^{\Delta V/\Delta V}$ (eB2 △V/△V) mutant mice was severely reduced reaching less than 25% of control tumors grown in wild type littermates (control). Astrocytoma tumors were stained with hematoxylin-eosin (HE). Scale bar, 1 mm. **b**, Astrocytomas tumors of ephrinB2$^{\Delta V/\Delta V}$ mice exhibited decreased vascularization as compared to wild type littermates (control) tumors. Higher magnification images of tumor vessels show vascular sprouts. Arrowheads point to filopodial extensions in the tumor vessels. Note the smooth and normalized vessels in the ephrinB2$^{\Delta V/\Delta V}$ mutants. Scale bars, 100 µm in a and 25 µm in b. Error bars represent SEM, Two-tailed t-test, * P<0.05. Reproduced with permission from Sawamiphak et.al., 2010.

endothelial tip cell function during guided migration of growing vessels in both developmental and pathological settings. In addition, the function of ephrinB2 as a regulator of VEGFR trafficking might not be restricted only to VEGFR2. VEGFR3, the major regulator of lymphangiogenesis whose role in active angiogenesis of blood vasculatures in both developmental and pathological settings has recently been revealed (Tammela et al., 2008), has been shown to internalized from the plasma membrane in an ephrinB2-dependent manner (Wang et al.). Blockade of ephrinB2 signaling to simultaneously interfere both with VEGFR2 and VEGFR3 functions might therefore represent an intriguing alternative of anti-angiogenic treatment for tumor therapy.

References

Adams, R.H., Diella, F., Hennig, S., Helmbacher, F., Deutsch, U., and Klein, R. (2001). The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* **104**, 57-69.

Adams, R.H., and Eichmann, A. Axon guidance molecules in vascular patterning. Cold Spring Harb Perspect Biol 2, a001875.

Bashaw, G.J., and Klein, R. Signaling from axon guidance receptors. *Cold Spring Harb Perspect Biol* **2**, a001941.

Benedito, R., Roca, C., Sorensen, I., Adams, S., Gossler, A., Fruttiger, M., and Adams, R.H. (2009). The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* **137**, 1124-1135.

Carmeliet, P., and Tessier-Lavigne, M. (2005). Common mechanisms of nerve and blood vessel wiring. *Nature* **436**, 193-200.

Dorrell, M.I., and Friedlander, M. (2006). Mechanisms of endothelial cell guidance and vascular patterning in the developing mouse retina. *Prog Retin Eye Res* **25**, 277-295.

Egea, J., and Klein, R. (2007). Bidirectional Eph-ephrin signaling during axon guidance. *Trends Cell Biol* **17**, 230-238.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., et al. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* **161**, 1163-1177.

Gould, G.W., and Lippincott-Schwartz, J. (2009). New roles for endosomes: from vesicular carriers to multi-purpose platforms. *Nat Rev Mol Cell Biol* **10**, 287-292.

Hellstrom, M., Phng, L.K., Hofmann, J.J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A.K., Karlsson, L., Gaiano, N., et al. (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* **445**, 776-780.

Howe, C.L., Valletta, J.S., Rusnak, A.S., and Mobley, W.C. (2001). NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway. *Neuron* **32**, 801-814.

Jekely, G., Sung, H.H., Luque, C.M., and Rorth, P. (2005). Regulators of endocytosis maintain localized receptor tyrosine kinase signaling in guided migration. *Dev Cell* **9**, 197-207.

Lu, X., Le Noble, F., Yuan, L., Jiang, Q., De Lafarge, B., Sugiyama, D., Breant, C., Claes, F., De Smet, F., Thomas, J.L., et al. (2004). The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature* **432**, 179-186.

Makinen, T., Adams, R.H., Bailey, J., Lu, Q., Ziemiecki, A., Alitalo, K., Klein, R., and Wilkinson, G.A. (2005). PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. *Genes Dev* **19**, 397-410.

Mukouyama, Y.S., Shin, D., Britsch, S., Taniguchi, M., and Anderson, D.J. (2002). Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* **109**, 693-705.

Olsson, A.K., Dimberg, A., Kreuger, J., and Claesson-Welsh, L. (2006). VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* **7**, 359-371.

Palamidessi, A., Frittoli, E., Garre, M., Faretta, M., Mione, M., Testa, I., Diaspro, A., Lanzetti, L., Scita, G., and Di Fiore, P.P. (2008). Endocytic trafficking of Rac is required for the spatial restriction of signaling in cell migration. *Cell* **134**, 135-147.

Siekmann, A.F., and Lawson, N.D. (2007). Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* **445**, 781-784.

Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S., and Di Fiore, P.P. (2008). Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev Cell* **15**, 209-219.

Sorkin, A., and von Zastrow, M. (2009). Endocytosis and signalling: intertwining molecular networks. *Nat Rev Mol Cell Biol* **10**, 609-622.

Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellstrom, M., Schomber, T., Peltonen, R., et al. (2008). Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature* **454**, 656-660.

Wang, Y., Nakayama, M., Pitulescu, M.E., Schmidt, T.S., Bochenek, M.L., Sakakibara, A., Adams, S., Davy, A., Deutsch, U., Luthi, U., et al. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature* **465**, 483-486.

Acknowledgement

My sincere gratitude goes to Prof. Amparo Acker Palmer for her invaluable scientific inputs, encouragement and constant support. I am also thankful to Dr. Alessandro Filosa and Prof. Ruediger Klein, whose constructive comments and generous support of experimental materials greatly help to shape up this work.

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Oct 2010-Present	Postdoctoral Fellow at the University of California San Francisco, in the group of Prof. Didier Stainier
Oct 2008 – Jun 2010	PhD student position at the Institute of Cell Biology and Neuroscience, Goethe University, Frankfurt, in the group of Prof. Amparo Acker-Palmer
Oct 2005 – Sep 2008	PhD student position at the Max Planck Institute of Neurobiology, Martinsried, in the group of Prof. Amparo Acker-Palmer
Nov 2004 – Mar 2005	Guest scientist at Kyorin University, Tokyo, in the group of Prof. Yoshikatsu Kanai
Jun 2002 – Mar 2004	Master student at Mahidol University, Bangkok (Master Degree of Science, Major: Molecular Biotechnology), in the group of Dr. Chuenchit Boonchird
Jun 1998 – Mar 2002	Bachelor student at Kasetsart University, Bangkok (Bachelor Degree of Science; Major: Genetics), in the group of Dr. Surin Piyachokanakul

Protokoll der Mitgliederversammlung 2011 der Deutschen Gesellschaft für Zellbiologie e.V. (DGZ)

Versammlungsort:
Universität Bonn, Raum/Hörsaal HS1
Versammlungstag: Donnerstag, 31. März 2011
Beginn: 19.00 Uhr, Ende: 19.45 Uhr

Anwesend sind insgesamt 30 Teilnehmer: 27 Mitglieder und 3 amtierende Vorstandsmitglieder (Prof. Dr. Harald Herrmann-Lerdon/Präsident, Prof. Dr. Ralph Gräf/Geschäftsführer und Prof. Dr. Eugen Kerkhoff/Sekretär).

TOP Jahresbericht des Präsidenten

Prof. Dr. Harald Hermann beginnt den Jahresrückblick mit einem Nachruf auf den im letzten Jahr völlig unerwartet verstorbenen ehemaligen Vizepräsidenten der DGZ Prof. Dr. Jürgen Wehland.

Im Anschluss berichtet Herr Prof. Herrmann über die DGZ-Jahrestagung 2010 in Regensburg. Die Jahrestagung in Regensburg hat einen Gewinn von etwa 55.000 Euro erwirtschaftet. Aufgrund der daraus resultierenden hohen finanziellen Rücklagen hat sich der Vorstand zur Abklärung steuerrechtlicher Fragen an den Steuerberater Herrn Kaiser aus Regensburg gewendet, der ein Experte für Steuer- und Vereinsrecht ist. Dieser Gewinn ermöglicht weitere Aktivitäten der Gesellschaft (s.u.).

Bezüglich der Organisation der Jahrestagungen wurde für die Jahrestagung 2011 in Bonn mit Conventus Congressmanagement & Marketing GmbH eine neue Organisationsfirma beauftragt. Für die nächste Jahrestagung, die 2012 in Dresden stattfindet und von Prof. Dr. Elisabeth Knust, Prof. Dr. Marino Zerial und Dr. Ewa Paluch organisiert wird, soll aber wieder mit der Firma MCI zusammengearbeitet werden, da erst die Erfahrungen mit Conventus hinsichtlich des Kongresses 2011 in Bonn ausgewertet werden sollen.

2010 hat die DGZ mit dem „International Meeting on Actin Dynamics" in Jena ein neues Meeting-Format eingeführt, das in den nächsten Jahren mit weiteren internationalen Tagungen zu ausgewählten Themen der Zellbiologie weitergeführt werden soll. 2011 findet in Potsdam das International Meeting mit dem Thema „The Spider´s Web: How Microtubule Organize Cellular Space" statt.

Die DGZ strebt zur Förderung der Zellbiologie in Deutschland an, die Organisation von wissenschaftlichen Tagungen auszubauen. Neben der Jahrestagung und dem International Meeting ist dabei auch die Nachwuchswissenschaftler-Tagung der DGZ ein fester Bestandteil. Sie findet in diesem Jahr in Jena zum Thema „RNA and Disease" statt.

Als weitere wichtige Fördermaßnahme wird die Modernisierung der DGZ-Zeitschrift angesehen. Die Zeitschrift wird zukünftig viermal im Jahr in englischer Sprache erscheinen. Der Titel wurde von „Zellbiologie aktuell" in „Cell News" geändert.

Weiterhin hat Herr Prof. Herrmann die Vergrößerung des Beirats der DGZ um zwei neue Mitglieder, Prof. Dr. Anne Spang (Basel) und Dr. Sylvia Erhardt (Heidelberg), bekannt gegeben. Dies wurde für notwendig erachtet, da bei den Beiratssitzungen nicht immer alle Mitglieder anwesend sein können und mit einem vergrößerten Beirat soll sichergestellt sein, dass immer eine differenzierte Diskussion und Meinungsbildung möglich ist.

BILANZ 2010
Einnahmen/Ausgaben-Zusammenstellung

Einnahmen	EUR
Mitgliedsbeiträge	45.344,18
Spenden u.a. Preisgelder	19.864,94
Zinsen	1.989,13
DGZ-Zeitschrift ZAK, Firmen-Links	13.611,10
DGZ-Tagungen	71.791,79
Überträge	35.994,54
Sonstige	0,00
Summe der Einnahmen	188.595,68
Guthaben am 31.12.2009:	257.959,05
DGZ:	186.776,13
Werner Risau Preis:	71.182,92

Ausgaben	EUR
Bankkosten	879,24
Retoure/Mitgliedsbeiträge	996,00
Reisekosten	6.755,68
Spenden	27.586,94
DGZ-Zeitschrift ZAK	33.598,65
DGZ-Tagungen	35.820,57
Bürokosten u.a. Gehalt Sekretärin, Homepage	72.953,46
Überträge	35.994,54
Sonstige	8.830,01
Summe der Ausgaben	223.415,09
Guthaben am 31.12.2010:	**223.139,64**
DGZ:	155.469,25
Werner Risau Preis:	67.670,39

Die Einnahmen und Ausgaben wurden durch die Kassenprüfer Frau Marie-Christine Dabauvalle und Herrn Hans-Georg Mannherz am 17. März 2011 in Heidelberg geprüft und für richtig befunden.

TOP Geschäfts-/Kassenbericht

Der Geschäftsführer erläutert die Finanzlage der Gesellschaft anhand der Einnahmen- und Ausgaben-Bilanz 2010 und stellt die Entwicklung der Mitgliederzahlen dar.

Zur Mitgliederentwicklung in 2010 berichtet der Geschäftsführer, dass wir 63 neue Mitglieder gewinnen konnten. Dem gegenüber stehen 53 verlorene Mitglieder: 46 Mitglieder kündigten, 6 Mitglieder mussten wegen nicht erfolgter Beitragszahlungen satzungsgemäß aus der Datenbank gelöscht werden und ein Mitglied ist leider verstorben. Seit Jahresbeginn 2011 bis zur Jahrestagung haben sich bereits 44 neue Mitglieder angemeldet.

TOP Bericht der Kassenprüfer

Der Geschäftsführer informiert, dass die Einnahmen und Ausgaben für das Kassenjahr 2010 durch die beiden Kassenprüfer Frau Marie-Christine Dabauvalle und Herrn Hans-Georg Mannherz am 17. März 2011 in Heidelberg geprüft und für richtig befunden wurden. Der Steuerberater, Herr Kaiser, war zugegen und begleitete die Kassenprüfung beratend.

TOP Entlastung des Vorstandes

Frau Prof. Brigitte Jockusch (TU Braunschweig) bringt den Antrag zur Entlastung des Vorstandes ein, der ohne Gegenstimmen bei 3 Enthaltungen der Vorstandsmitglieder angenommen wird.

TOP Verschiedenes

Frau Prof. Brigitte Jockusch stellt die Modernisierung und Umbenennung der DGZ-Zeitschrift in „Cell News“ im Hinblick auf den Verfall der Deutschen Sprache in der Wissenschaft zu Diskussion. Es besteht ein großer Konsens, dass die Herausgabe der Zeitschrift in englischer Sprache ein wichtiger Schritt ist, um die Aktivitäten der Deutschen Zellbiologen international zu vernetzen und sichtbar zu machen.

Herr Prof. Dr. Ralph Gräf bittet um Verbesserungsvorschläge und Anregungen zum Internetauftritt der DGZ.

Der Präsident schließt die Versammlung.

Prof. Dr. Harald Herrmann-Lerdon
(Präsident)
Prof. Dr. Eugen Kerkhoff
(Sekretär)

Busy orbits around the centrosome

Felix Bärenz, Dmytro Mayilo and Oliver J. Gruss

Introduction

Since its first description by Theodor Boveri in 1888, the centrosome has been studied intensely, so that we now have detailed information about its structure, molecular composition and its various functions.

The centrosome consists of two centrioles, which generally appear in electron microscopy as barrel-shaped structures usually composed of nine triplet microtubules. An amorphous mass of pericentriolar material surrounds the centrioles and accumulates many proteins important for integrity and function of centrosomes, such as the γ-tubulin ring complex that mediates microtubule nucleation and capping. In animal somatic cells, the centrosome generally accounts for the major microtubule organising center (MTOC), and the duplicated pair of centrosomes determine the poles of the microtubule-based mitotic spindle. Despite the detailed insights into the centrosome's structure and function, it has been a complete mystery until a few years ago how centrosomes duplicate and assemble. Moreover, it is still largely unclear if and how centrosomal proteins or protein complexes are exchanged, replaced or qualitatively altered. Previously identified cytoplasmic granules, named "pericentriolar" or "centriolar satellites", might fulfil such functions in protein exchange and communication between the centrosomes and the cytoplasm. In this review, we summarize current knowledge about structure, molecular composition and possible roles of the satellites that seem to environ the core of the centrosome in most animal cells.

Identification of granules surrounding the centrosome

Almost all aspects of centrosomal functions were still undetermined during the beginning of the second half of 20-th century when several groups exploited electron microscopy (EM) to analyse the ultrastructure of the centrosome. EM not only revealed centriole architecture but also identified electron dense granules of around 70-100 nm in diameter localizing around the centrosome in various cell types. The composition and function of these obscure granules was unknown at that time (de Thé, 1964) although in 1964 de Thé and colleagues noted that the granules were associated with microtubules originating from the centrosome (de Thé, 1964), and that the amount of the granules varied throughout the cell cycle (Rattner, 1992). Similar structures were independently entitled "fibrous granules" when observed around replicating centrioles during and even after assembly of the numerous basal bodies required for motile cilia assembly in epithelial cells (Dirksen, 1991).

PCM-1: localization to pericentriolar satellites and its functional implications for the centrosome

In 1994 Balczon et al. discovered and initially described PCM-1 (pericentriolar material 1) as a centrosome-associated protein in interphasic HeLa cells (Balczon et al., 1994). Although PCM1 levels remain unchanged during the cell cycle, the protein alters its association state with the centrosome: it dissociates from centrosomes in early G2 phase before it further reaccumulates in the vicinity of and on the centrosome again in G1 phase indicating dynamic exchange of centrosome targeted PCM1 (Balczon et al., 1994). Importantly, further studies confirmed that PCM-1 localizes to cytoplasmic granules around the centrosome in Xenopus A6 and mouse Eph4 cells (Kubo et al., 1999), Fig. 1. On the basis of data available at that time, it was proposed that PCM-1 may act as a scaffold protein for these and other previously observed cytoplasmic granules, which were then collectively named "centriolar" or "pericentriolar satellites" (Kubo et al., 1999).

When live imaging techniques became generally available, it was quickly realised that centriolar satellites move along microtubules in minus end direction towards the centrosome and accumulate there (Balczon et al., 1999; Kubo et al., 1999). The movement of PCM-1 containing centriolar satellites turned out to depend on dynein, but did not seem to involve kinesin-mediated transport (Kubo et al., 1999). In line with this, PCM-1 co-purified with microtubules from CHO cell lysates and immunodepletion of dynein from CHO extracts significantly decreased the amount of microtubule-associated PCM-1. On the other hand, immuno-depletion of kinesin using specific antibodies had no effect on the microtubule association of PCM-1 (Balczon et al., 1999).

Despite the fact that PCM-1 seemed not to be a core centrosomal component, loss of function experiments on PCM-1 indicated a role in the assembly of centrin, pericentrin and ninein (Dammermann and Merdes, 2002). A possible function of PCM1 associated satellites could be to mediate transport of proteins of both centriolar and pericentriolar material from the cytoplasm to the centrosome along microtubules (Fig. 1 and 2). Thereby PCM-1 itself possibly functions as a carrier-protein (Dammermann and Merdes, 2002). The need

for correct delivery of centrosomal proteins to the centrosome is most obvious during centrosome duplication when new proteins for centriole replication and duplication of pericentriolar material have to be available at the centrosome. Apart from that centrosomal satellites may be particularly important for regulated changes of the protein composition of centrosomes during developmental decisions when the centrosome adapts for functions in e.g. nuclear migration or spindle positioning. Consistent with a rapidly changing repertoire, many "centrosomal" proteins are not strictly localized to the centrosome, but are also present in a cytoplasmic pool. Cytoplasmic factors that function in correct folding and targeting of these centrosomal proteins can strongly influence the equilibrium between centrosome-associated and free centrosomal proteins. PCM-1 containing granules may provide centrosomal proteins with a favourable environment for proper folding and maintenance of biological activity before they are finally incorporated into centrosomes (Dammermann and Merdes, 2002; Hames et al., 2005), Fig. 2.

The protein ninein was originally identified as a component of the pericentriolar material before Mogensen et al. demonstrated that ninein mislocalization still allows daughter centrioles to nucleate microtubules, but fails to anchor them. More recently, it was shown that PCM-1 depletion leads to mislocalization of ninein and thereby to the loss of microtubule anchorage (Dammermann and Merdes, 2002), Fig. 2. Centriolar satellites and associated PCM-1 therefore seem to be involved in microtubule anchorage by targeting ninein to the centrosome (Dammermann and Merdes, 2002). This is consistent with the previous publication by Clark and Mayer that the correct targeting of microtubule anchorage factors is dependent on the dynein-dynactin complex. They showed that dynactin inhibition has a direct effect on microtubule organization at the centrosomal region (Clark and Meyer, 1999). Like ninein, Pericentrin gets recruited to the centrosome in a PCM-1 granule dependent manner although its mislocalization does not interfere with centrosome mediated microtubule organization.
Satellite proteins were also shown to be required to maintain the nucleation capacity of centrosomes. Oshimori et al. characterised CEP-72 as a binding partner of the previously indentified Kizuna protein. Both proteins turned out to localise around the core of the centrosome in centrosomal satellites (Table I). Strikingly, knockdown of CEP-72 led to decreased levels of γ-TURC at the centrosome. The resulting reduction in nucleation capacity of centrosomes as well as an apparently reduced stability of spindle poles interfered with proper mitotic aster assembly and led to compromised spindle assembly (Oshimori et al., 2009). The polarity gene Par6α also turned out to be a satellite protein (Table I), and its knockdown in human HeLa cells caused severe mitotic defects and the frequent assembly of multipolar spindles. Par6α depleted cells displayed reduced levels of ninein, PCM1 and centrin at the centrosomes (Kodani et al., 2010). Interstingly, it could be shown that Par6α also interacts with the dynein complex component $p150^{glued}$. Par6α may therefore be a critical mediator of dynein-driven transport of satellite proteins to the centrosome. CEP-90, another sa-

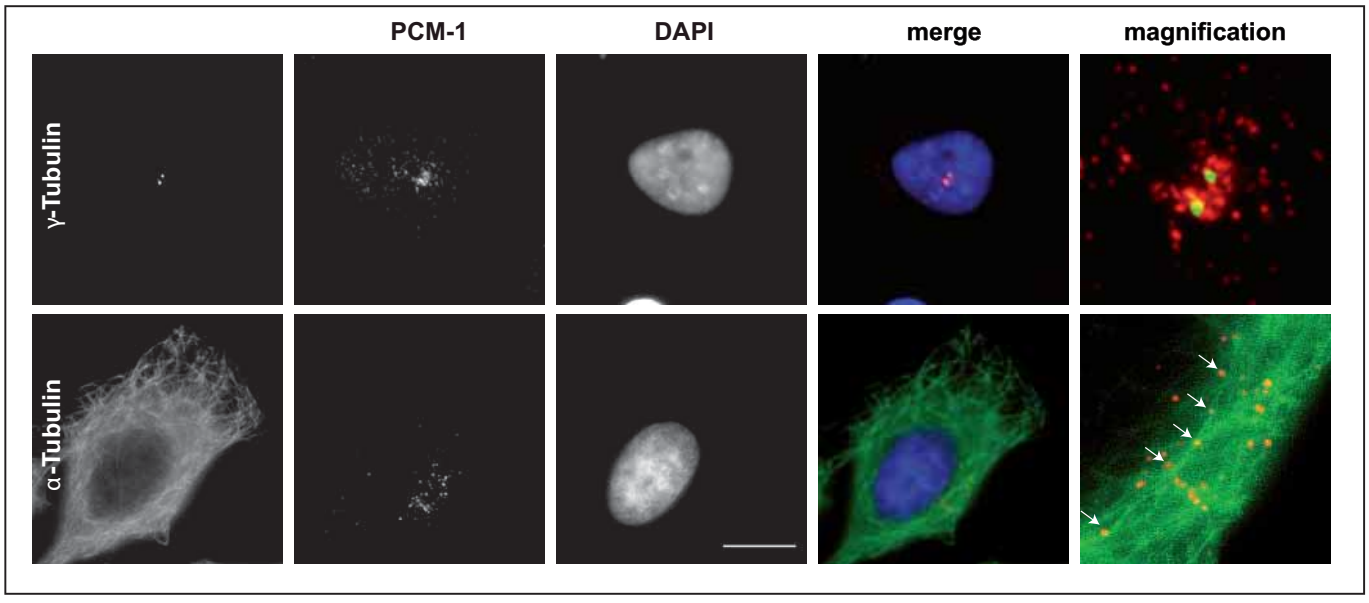

Fig.1 *Immunofluorescence micrograph of human RPE cells, in which the satellite protein PCM-1 (red in merge) was visualised by indirect immunofluorescence. DAPI stains the DNA (blue in merge), specific tubulin antibodies (green in merge) visualise γ-tubulin (upper panels) or α-tubulin (lower panels). Scalebar: 10 µm.*

tellite protein, was recently reported to have a function in mitosis (Table I). Knock-down of the protein caused spindle pole fragmentation and misalignment of the chromosomes in metaphase (Kim and Rhee, 2011). Kim and Rhee proposed that the observed defects in spindle pole maintenance could be due to the role of CEP-90 as a scaffold to ensure the structural integrity of spindle poles against the physical forces of the attached spindle microtubules. In this model, CEP-90 may function in early prophase as a mitosis specific adaptor of other satellite proteins or centrosomal regulators that are required for spindle pole integrity during mitosis (Kim and Rhee, 2011).

Further centrosome-communicative functions of the PCM-1 satellite complex may be required in the process of centrosome separation: Nek2 kinase, involved in the regulation of centrosome structure at the G2/M transition, was shown to partially colocalize with PCM-1 containing centrosomal satellites (Hames et al., 2005). Nek2 overexpression led to the loss of PCM-1 containing satellites and, in turn, the depletion of PCM-1 reduced the abundance of Nek2 at the centrosome. Although PCM-1 turned out to be not per se required for the assembly of Nek2 at the centrosome, these data suggest a link between the integrity of centrosomal satellites and Nek2 function at centrosomes (Hames et al., 2005).

PCM1 function in embryonic neural progenitors

Centrosomes have an important role in nucleating, anchoring and organizing microtubules, in cell cycle progression as well as cell polarization and ciliogenesis. These functions are particularly critical for proper neurodevelopment, especially in the cerebral cortex (Thornton and Woods, 2009). Recent studies implicated that the risks for various neuropsychiatric diseases, such as schizophrenia and bipolar disorder are higher when centrosomal functions are disturbed. These disorders have been associated with compromised functions of the centrosomal satellite components PCM-1, the Bardet-Biedl syndrome 4 protein (BBS4) and the *disrupted in schizophrenia 1* gene product (DISC1), see Table I. DISC1 as well as BBS4 were found to interact with PCM-1 (Kim et al., 2004). It was demonstrated that DISC1 plays an important role in cell differentiation, proliferation, as well as in axon and dendrite out-growth and, most recently, for a switch of mammalian neuronal progenitor cells from proliferative activity to migrating behaviour (Ishizuka et al., 2011).

Mutation or misregulation of BBS4 is one of the causes of BBS, an inherited disorder characterized by renal dysfunction, obesity, polydactyly and diverse neuropsychiatric symptoms (Katsanis, 2004). Kim et al. reported that BBS4 is a pericentriolar matrix protein, which has a crucial role in cargo recruitment to centrosomal satellites and may interact with PCM1. BBS4 depletion led to dispersion of PCM-1 into the cytoplasm, microtubule disorganization, cell cycle alterations and apoptosis (Kim et al., 2004). These observations suggest a direct interaction of PCM-1 and BBS4. BBS4 was shown to additionally interact with $p150^{glued}$, promoting the idea of BBS4 as a dynein associated adaptor molecule in PCM1 mediated transport of centrosomal satellites (Kim et al., 2004).

Recently, DISC1 was reported to communicate with several BBS proteins (Kamiya et al., 2008) and PCM1, DISC1 and BBS4 were shown to interact with each other through defined binding domains (Kamiya et al., 2008). These findings argue that DISC1 and BBS4 together help to target PCM1 and associated centrosomal cargos in a synergistic manner to the centrosome (Kamiya et al., 2008). Again, the depletion of DISC1, BBS4 or PCM-1 triggered the formation of neuronal migration defects in embryonic neuronal tissues.

It seems that pericentriolar satellite mediated trafficking of centrosomal proteins is essential for proper functioning of the centrosome in particular in neuronal progenitors. It was consistently shown that trafficking of centrosomal satellites in neuronal progenitors relies on the interaction between PCM-1 and Hook3 (Ge et al., 2010): Hook3 is the mammalian ortholog of *C. elegans* ZYG-12 that links the centrosome to the nucleus. Hook3 turned out to be a centrosomal satellite protein (Table I) and interfering with Hook3 / PCM-1 interaction compromised assembly of new protein material at the centrosome. At the cellular level, this impaired a process called interkinetic nuclear migration (INM), in turn leading to overproduction of neurons at the expense of neuronal stem cell pool (Ge et al., 2010). INM was first reported in 1935 describing the oscillation of neuronal progenitor nuclei to and back from their apical domains (ventricular surface) during the cell cycle. Before undergoing mitosis, the neuronal progenitor nuclei are transported to the very apical side, where mitosis and cytokinesis take place. The stereotypically apical cell divisions govern the balance between differentiation and selfrenewal of neuronal progenitors. A disruption of INM consistently leads to cell division away from the ventricular surface resulting in the formation of two daughter cells, which start to differentiate into neurons. This in turn causes premature loss of neuronal progenitors. Dissociation of the centrosome from the nucleus, or detachment of microtubules from the centrosome due to the lack of microtubule anchorage proteins like ninein, impairs nuclear movement during INM. This interferes with maintenance of the neuronal stem cells pool and causes severe diseases like bipolar disorder and schizophrenia. Likewise, compromised INM has been shown recently as a result of loss of function of Lis1 (Gambello et al., 2003) or the centrosomal proteins CEP-120 and TACC (Xie et al., 2007). All in all these findings underline the importance of the centrosome in the regulation of neuronal stem cell pool maintenance.

Functional studies on CEP-290 recently confirmed the strong link between key centrosomal functions and neurogenesis. Mutations in CEP-290 lead to the formation of *Joubert Syndrome* (JS). JS is a developmental brain disorder characterized by cerebellar vermis hypoplasia, abnormal eye movement, ataxia and mental retardation. Recent data documented that CEP-290, like BBS4, localizes to centrosomes and directly binds to PCM-1 although CEP-290 was never detected in the scattered cytoplasmic granules containing PCM-1 (Table I). CEP-290 depletion led to disorganization of the microtubule network similar to the observed knockdown-phenotype of other PCM-1 interaction partners (Kim et al., 2008). In contrast to BBS4 depletion, which resulted in PCM-1 dispersion, the depletion of CEP-290 caused abnormally increased concentric accumulation of PCM-1 at the centrosome. These observations suggest that CEP-290 might function in the destabilization of the PCM-1-dynein motor interaction. Another possibility is that CEP-290 promotes PCM-1 association with kinesin motors in order to drive the movement of the complex back to the cytoplasm. Recent findings support this idea since CEP-290 was shown to interact not only with dynein, but also with kinesin motors (McEwen et al., 2007).

Pericentriolar satellites in Ciliogenesis

As mentioned above, centrosomal satellites were first observed in ciliated epithelial cells that assembled a large number of basal bodies for motile cilia formation. BBS4 may be the paradigm of overlapping protein functions between centrosomal satellites and basal body assembly in ciliogenesis but more proteins underline this general principle. To date, there are 12 gene products known to be involved in BBS. Mutations in each of the genes cause similar pathologies suggesting that all BBS gene products function in the same molecular pathway. BBS proteins localize primarily to the centrosome and at the basal body of ciliated cells. Cilia formation is a highly regulated multistep process that includes the docking of the basal body to the cell cortex, elongation of the cilium membrane and the axoneme, and the recruitment of signalling molecules (Gruss, 2010). Together, some of the disease-defined BBS proteins form a multisubunit complex, the BBSome that has essential functions in ciliogenesis (Loktev et al., 2008; Nachury et al., 2007). It was shown to act in membrane extension by activation of Rab8 and is also involved in microtubule acetylation, a hallmark of ciliogenesis (Loktev et al., 2008). Several ciliopathy disease proteins were recently documented to be localized to satellites, including CEP-290 (Kim et al., 2008; Kim et al., 2004), which was detected at basal bodies of

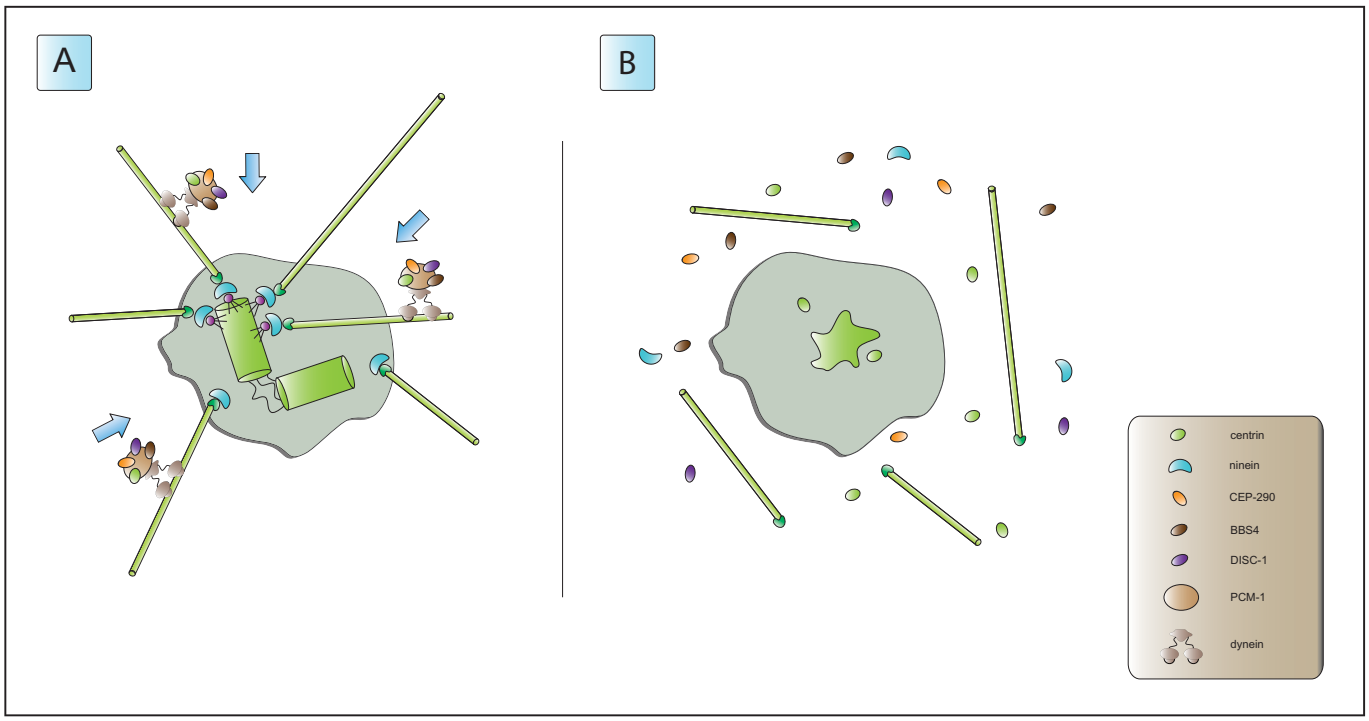

Fig.2 *Schematic drawing of centrosomal satellites under normal conditions (A) and after knock-down of PCM-1, the first identified satellite protein (B): Known components of satellites such as BBS4, DISC1 or CEP-290 associate with PCM-1 to promote stabilisation and dynein-mediated targeting of core centrosomal proteins such as centrin and ninein. Loss-of-fuction of PCM-1, or other satellite proteins, lead to structural defects of centrosomes that e.g. loose their function as microtubule organising centers.*

ciliated cells and to the connecting cilium of retinal photoreceptors (Valente et al., 2006). A mouse CEP-290 k.o. cell line displayed mislocalization of both photo transduction proteins and odorant-signaling proteins in the cilia of olfactory receptor neurons (Valente et al., 2006). Consistent with that, knockdown experiments in human cells revealed perturbed BBSome assembly upon CEP-290 downregulation (Kim et al., 2008).

Likewise, the recently described FOR20 protein links centrosomal satellites and ciliogenesis (Sedjai et al., 2010), (Table I). Depletion of FOR20, as shown for other satellite members, led to PCM-1 dispersion. On the other hand PCM-1 depletion caused FOR20 to be exclusively localized to the centrosome while its satellite pool is lost (Sedjai et al., 2010). FOR20 depletion affected cilia morphology qualitatively resulting in much shorter cilia compared to wild type (Sedjai et al., 2010). This suggests that satellite disruption in general affects the recruitment of proteins to the basal body (Sedjai et al., 2010).

Another protein linking satellites with maturating cilia is the disease-related *oral facial syndrome protein 1*, OFD1. The-oral-facial (OFD) syndromes share dysmorphology of the mouth, face and digits. OFD type 1 was first described in 1954 by Papillon-League and Psaune as an x-linked dominantly inherited disease with prenatal lethality in affected males. Females born with OFD dysmorphology regularly develop polycystic kidneys. The syndrome related to loss-of-function of OFD1 represents a classical ciliopathy, as the OFD1 protein is strictly required for primary cilia formation (Singla et al., 2010). OFD1 protein localises to the basal body of cilia, but is also present in centrosomal satellites (Table I). Furthermore, OFD1 was shown to colocalize with BBS4 and CEP-290 (Lopes et al., 2011). The consistent observations that ciliopathy proteins like BBS4, CEP-290 and OFD1 are also localized at centrosomal satellites underline the importance of the satellites in diverse centrosome related processes including ciliogenesis. Finally, this may indicate that some possible satellite functions, such as mediating protein targeting to the centrosome, and communication of the centrosome with the surrounding cytoplasm, are required for ciliogenesis. Nevertheless, the pool of factors involved in satellite functions on the one hand, and cilia assembly on the other hand, is by no means identical, strikingly documented by the fact that the first identified centrosomal satellite component, PCM1, seems not to be required for ciliogenesis.

Protein name	localization	Interaction partners co-localizations	Potential function	Molecular weight (kDa)
BBS4	Centrosomal satellites, cilia basal body	BBS- proteins, $p150^{glued}$, PCM-1, DISC-1	PCM-1 and cargo recruitment to pericentriolar satellites, neuronal migration	58
CEP-72	Centrosomal satellites, centrosome	Kizuna	Targeting factor for γ-TURC and Kizuna, required for mitotic spindle pole integrity	72
CEP-90	Centrosomal satellites		Scaffold that ensures the structural integrity against the physical forces of the attached spindles	90
CEP-290	Centrosome, basal-body and centrosomal satellites (?)	PCM-1, Dynein, Kinesin	Cellular transport, destabilizing PCM-1 dynein interaction, promote Kinesin mediated transport of pericentriolar satellites	290
DISC-1	Centrosomal satellites	BBS-proteins, BBS4, PCM-1, Kendrin	PCM-1 and cargo recruitment to pericentriolar satellites, neuronal migration	94
FOR-20	Centrosomal satellites, centrosome		PCM-1 localization and PCM-1 microtubule interaction, Ciliogenesis	20
Hook-3	Centrosomal satellites	PCM-1	Trafficking of centrosomal proteins, interkinetic nuclear migration	83
Kizuna	Centrosomal satellites, centrosome	CEP-72	Targeting factor for γ-TURC, required for mitotic spindle pole integrity	75
OFD-1	Centrosomal satellites	BBS4, CEP-290	Primary cilia formation	28
Par6α	Centrosomal satellites,	PCM1, $p150^{glued}$	Mediator of dynein driven transport of satellites to the centrosome	38
PCM-1	Centrosomal satellites, centrosome	BBS4, DISC-1, Dynein	Transport of centrosomal proteins	228

Table 1 *The table summarises proteins of centriolar satellites and states their potential function, interaction partners and additional localization.*

Concluding remarks

The discovery of proteins that are part of centrosomal satellites now gives us first insights into the function of these structures: they seem to serve as platforms for dynein and possibly kinesin mediated transport of proteins that in turn carry out key functions of the centrosome. Satellites therefore include proteins required for transport complex assembly itself (PCM1, BBS4, DISC1 etc.), as well as proteins that presumably act as cargo delivered to or transported away from the centrosome (centrin, pericentrin, ninein). The transport complex module might have additional functions in assembly and folding of the cargos. Interfering with the functions of transport complex proteins therefore results in defects of basic centrosomal functions, similar to the direct disruption of the core centrosomal cargo itself, i.e. improper microtubule anchoring and organization. This, in turn, affects essential developmental processes such as INM or spindle positioning.

Although the identification of key components of centriolar satellites gives us some clue into possible roles of these structures, many details still remain unclear. We do not understand at all, when, how and why satellites arise or dissociate. The complete functional proteome of centrosomal satellites is still elusive; for example, we do not know the molecular basis for their dynamic behaviour, as well as details about cell cycle regulated changes in composition, size and localization. In order to address these questions the reconstitution of satellite assembly and dynamics in cell free extracts (Kubo et al., 1999) has to be further developed. Xenopus egg extracts faithfully recapitulate centrosome function in aster formation and spindle assembly (Reber et al., 2008; Sawin et al., 1992). These extracts are even capable of assembling centrioles de novo (Eckerdt et al., 2011; Hatch et al., 2010). We recently used Xenopus extracts to analyse developmentally regulated microtubule binding proteins and found new, uncharacterised centrosomal and centrosomal satellite proteins (Bärenz et al. manuscript in preparation). In that respect, it seems an invaluable advantage of the embryonic frog extract that centrosomal proteins are obviously present as free proteins or protein subcomplexes ready to assembly into centrioles, pericentriolar material or centrosomal satellites. Assembly intermediates of centrosomal structures can therefore be analysed in proteomic detail. These details will provide insights into the mechanism of centrosome assembly, and may supply valuable knowledge about centrosome associated diseases thereby yielding new therapeutically relevant information.

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References

Balczon, R., Bao, L., and Zimmer, W.E. (1994). PCM-1, A 228-kD centrosome autoantigen with a distinct cell cycle distribution. *J Cell Biol* **124**, 783-793.

Balczon, R., Varden, C.E., and Schroer, T.A. (1999). Role for microtubules in centro-some doubling in Chinese hamster ovary cells. *Cell Motil Cytoskeleton* **42**, 60-72.

Clark, I.B., and Meyer, D.I. (1999). Overexpression of normal and mutant Arp1alpha (centractin) differentially affects microtubule organization during mitosis and in-terphase. *J Cell Sci* **112** (Pt 20), 3507-3518.

Dammermann, A., and Merdes, A. (2002). Assembly of centrosomal proteins and microtubule organization depends on PCM-1. *J Cell Biol* **159**, 255-266.

de Thé, G. (1964). Cytoplasmic Microtubules in Different Animal Cells. *J Cell Biol* **23**, 265-275.

Dirksen, E.R. (1991). Centriole and basal body formation during ciliogenesis revis-ited. *Biol Cell* **72**, 31-38.

Eckerdt, F., Yamamoto, T.M., Lewellyn, A.L., and Maller, J.L. (2011). Identification of a polo-like kinase 4-dependent pathway for de novo centriole formation. *Curr Biol* **21**, 428-432.

Gambello, M.J., Darling, D.L., Yingling, J., Tanaka, T., Gleeson, J.G., and Wynshaw-Boris, A. (2003). Multiple dose-dependent effects of Lis1 on cerebral cortical development. *J Neurosci* **23**, 1719-1729.

Ge, X., Frank, C.L., Calderon de Anda, F., and Tsai, L.H. (2010). Hook3 interacts with PCM1 to regulate pericentriolar material assembly and the timing of neu-rogenesis. *Neuron* **65**, 191-203.

Gruss, O.J. (2010). Nuclear transport receptor goes moonlighting. *Nat Cell Biol* **12**, 640-641.

Hames, R.S., Crookes, R.E., Straatman, K.R., Merdes, A., Hayes, M.J., Faragher, A.J., and Fry, A.M. (2005). Dynamic recruitment of Nek2 kinase to the centro-some involves microtubules, PCM-1, and localized proteasomal degradation. *Mol Biol Cell* **16**, 1711-1724.

Hatch, E.M., Kulukian, A., Holland, A.J., Cleveland, D.W., and Stearns, T. (2010). Cep152 interacts with Plk4 and is required for centriole duplication. *J Cell Biol* **191**, 721-729.

Ishizuka, K., Kamiya, A., Oh, E.C., Kanki, H., Seshadri, S., Robinson, J.F., Murdoch, H., Dunlop, A.J., Kubo, K., Furukori, K., et al. (2011). DISC1-dependent switch from progenitor proliferation to migration in the developing cortex. *Nature* **473**, 92-96.

Kamiya, A., Tan, P.L., Kubo, K., Engelhard, C., Ishizuka, K., Kubo, A., Tsukita, S., Pulver, A.E., Nakajima, K., Cascella, N.G., et al. (2008). Recruitment of PCM1 to the centrosome by the cooperative action of DISC1 and BBS4: a candidate for psychiatric illnesses. *Arch Gen Psychiatry* **65**, 996-1006.

Katsanis, N. (2004). The oligogenic properties of Bardet-Biedl syndrome. *Hum Mol Genet* **13** Spec No 1, R65-71.

Kim, J., Krishnaswami, S.R., and Gleeson, J.G. (2008). CEP290 interacts with the centriolar satellite component PCM-1 and is required for Rab8 localization to the primary cilium. *Hum Mol Genet* **17**, 3796-3805.

Kim, J.C., Badano, J.L., Sibold, S., Esmail, M.A., Hill, J., Hoskins, B.E., Leitch, C.C., Venner, K., Ansley, S.J., Ross, A.J., et al. (2004). The Bardet-Biedl protein BBS4 targets cargo to the pericentriolar region and is required for microtubule anchoring and cell cycle progression. *Nat Genet* **36**, 462-470.

Kim, K., and Rhee, K. (2011). The pericentriolar satellite protein CEP90 is crucial for integrity of the mitotic spindle pole. *J Cell Sci* **124**, 338-347.

Kodani, A., Tonthat, V., Wu, B., and Sutterlin, C. (2010). Par6 alpha interacts with the dynactin subunit p150 Glued and is a critical regulator of centrosomal protein recruitment. *Mol Biol Cell* **21**, 3376-3385.

Kubo, A., Sasaki, H., Yuba-Kubo, A., Tsukita, S., and Shiina, N. (1999). Centriolar satellites: molecular characterization, ATP-dependent movement toward cen-trioles and possible involvement in ciliogenesis. *J Cell Biol* **147**, 969-980.

Loktev, A.V., Zhang, Q., Beck, J.S., Searby, C.C., Scheetz, T.E., Bazan, J.F., Slusar-ski, D.C., Sheffield, V.C., Jackson, P.K., and Nachury, M.V. (2008). A BBSome subunit links ciliogenesis, microtubule stability, and acetylation. *Dev Cell* **15**, 854-865.

Lopes, C.A., Prosser, S.L., Romio, L., Hirst, R.A., O'Callaghan, C., Woolf, A.S., and Fry, A.M. (2011). Centriolar satellites are assembly points for proteins impli-cated in human ciliopathies, including oral-facial-digital syndrome 1. *J Cell Sci* **124**, 600-612.

McEwen, D.P., Koenekoop, R.K., Khanna, H., Jenkins, P.M., Lopez, I., Swaroop, A., and Martens, J.R. (2007). Hypomorphic CEP290/NPHP6 mutations result in anosmia caused by the selective loss of G proteins in cilia of olfactory sensory neurons. *Proc Natl Acad Sci* U S A **104**, 15917-15922.

Nachury, M.V., Loktev, A.V., Zhang, Q., Westlake, C.J., Peranen, J., Merdes, A., Slusarski, D.C., Scheller, R.H., Bazan, J.F., Sheffield, V.C., et al. (2007). A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. *Cell* **129**, 1201-1213.

Oshimori, N., Li, X., Ohsugi, M., and Yamamoto, T. (2009). Cep72 regulates the lo-calization of key centrosomal proteins and proper bipolar spindle formation. *EMBO J* **28**, 2066-2076.

Rattner, J.B. (1992). Ultrastructure of centrosome domains and identification of their protein components. In The Centrosome, V.I. Kalnins, ed. (San Diego, CA, Academic Press, Inc.), pp. 45-69.

Reber, S., Over, S., Kronja, I., and Gruss, O.J. (2008). CaM kinase II initiates meiotic spindle depolymerization independently of APC/C activation. *J Cell Biol* **183**, 1007-1017.

Sawin, K.E., LeGuellec, K., Philippe, M., and Mitchison, T.J. (1992). Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature* **359**, 540-543.

Sedjai, F., Acquaviva, C., Chevrier, V., Chauvin, J.P., Coppin, E., Aouane, A., Coulier, F., Tolun, A., Pierres, M., Birnbaum, D., et

al. (2010). Control of cilio-genesis by FOR20, a novel centrosome and pericentriolar satellite protein. *J Cell Sci* **123**, 2391-2401.

Singla, V., Romaguera-Ros, M., Garcia-Verdugo, J.M., and Reiter, J.F. (2010). Ofd1, a human disease gene, regulates the length and distal structure of centrioles. *Dev Cell* **18**, 410-424.

Thornton, G.K., and Woods, C.G. (2009). Primary microcephaly: do all roads lead to Rome? *Trends Genet* **25**, 501-510.

Valente, E.M., Silhavy, J.L., Brancati, F., Barrano, G., Krishnaswami, S.R., Castori, M., Lancaster, M.A., Boltshauser, E., Boccone, L., Al-Gazali, L., et al. (2006). Mutations in CEP290, which encodes a centrosomal protein, cause pleiotropic forms of Joubert syndrome. *Nat Genet* **38**, 623-625.

Xie, Z., Moy, L.Y., Sanada, K., Zhou, Y., Buchman, J.J., and Tsai, L.H. (2007). Cep120 and TACCs control interkinetic nuclear migration and the neural pro-genitor pool. *Neuron* **56**, 79-93.

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What can a single molecule teach us?

Zeynep Ökten

To cope with increasing infrastructural demands in highly compartmentalized cells, eukaryotes have evolved an intricate network of highways –F-actin and microtubules- and associated vehicles – aptly called motor proteins (Hirokawa et al., 2009; Spudich, 1994; Vale, 2003). Coupling force generation by these enzymes to directed, long-range transport on the cytoskeletal network is arguably one of the most crucial achievements in eukaryotic evolution. To maintain a viable infrastructure, hundreds of motors from three superfamilies are at work in any given eukaryotic cell. The energy needed to drive these processes is provided by the intracellular ATP. The kinetic adaptation that enables such long-range transport is termed *processivity*. Processive motors are capable of taking multiple, unidirectional "steps" as a single molecule without dissociating from their respective filaments. Kinesin and dynein family of molecular motors support long-range transport on microtubules. First identified in the axons of the giant squid some 25 years ago, the double-headed kinesin-1 is capable of taking hundreds of steps per encounter with the microtubule and serves as a paradigm of processive motors. The myosin family of molecular motors are involved in the actin-based transport, with the double-headed myosin V being the prototype of processive myosins (Mehta et al., 1999).

Advent of single molecule analysis and its significance for the motor field

In the past two decades, scientific inquiry of biological processes has reached an unprecedented level of depth with the onset of state of the art single molecule techniques. Manipulation of biological materials at single molecule level unmasked ensemble pathways and allowed invaluable insights that are concealed in bulk assays. The field of molecular motors in particular benefited immensely from these technical advances. It has been almost two decades since the stepping of a single kinesin motor was directly visualized on nanometer scale using optical tweezers (Svoboda et al., 1993). In its simplest form, an optical tweezers uses the force of a strongly focused laser light to stably trap a micron-sized object, e.g. a spherical polystyrene bead as illustrated in Figure 1.

Kinesins on trapped beads

Trapped polystyrene beads sparsely coated with motor proteins are readily amenable to experimental manipulation (Figure 1). In a flow chamber with surface-bound microtubules and under saturating ATP conditions, a single motor molecule starts to walk along the filament, pulling the bead out of the laser-focus, which exerts a restoring force similar to a mechanical spring (Figure 1, left panel). While the motor walks, the restoring force increases until the motor detaches from the filament and the

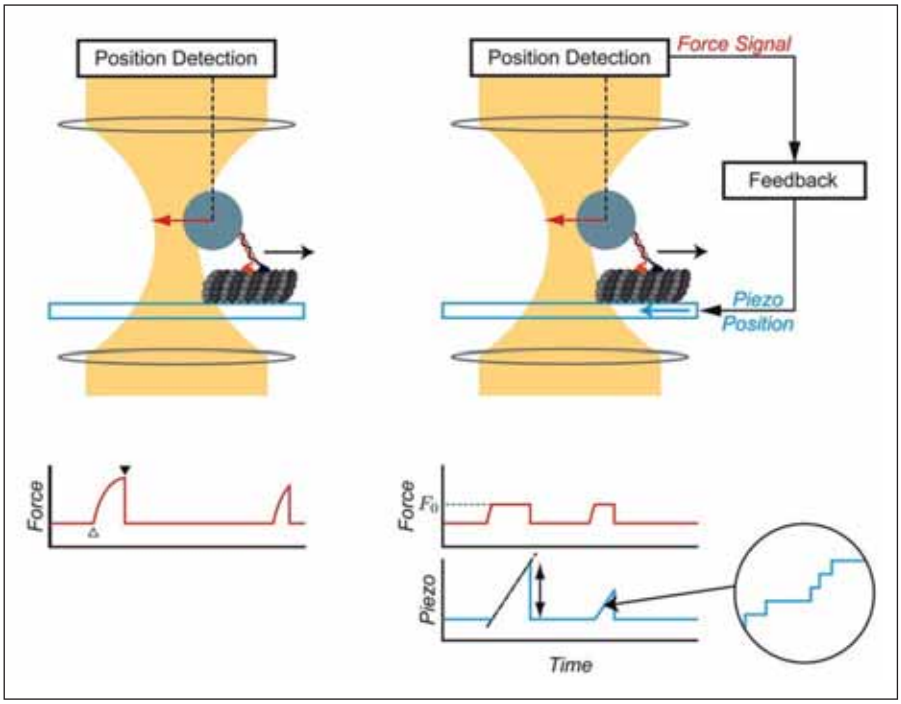

Figure 1: Illustration of the experimental set-up. Left Panel depicts the stationary trapping of a polystyrene bead (blue) that is decorated with motor proteins in a focused laser beam (yellow). The optical trap exerts a force on the bead x is the displacement of the bead. The red curve illustrates 'walking events' displayed by a single motor attached to a trapped bead under load. **Right Panel** illustrates the force feed-backed trapping mode. The restoring force acting on the motor is kept constant by moving the piezo stage in the opposite direction to the motor's movement. Contrary to the stationary modus, the piezo signal (blue) gives information about the run length of a motor and allows resolving discrete steps at limiting ATP concentrations.

bead relaxes back to the centre of the focus. Eventually the motor will start another run along the filament. The red curve represents a typical trace from a single motor recorded under the above-mentioned conditions (Figure 1, left panel). The key biophysical parameters obtainable from these measurements are the *stall force* and the *motor's activity*, respectively. The typical stall force of a kinesin motor is around 6 picoNewton which corresponds to the value on the y-axis when a walking molecule lets go from its track as indicated by the black triangle (Figure 1, left panel). The frequency of the walking events displayed by the motor is another convenient measure reporting on its single molecule activity.

This basic setup can be extended by a feedback loop which allows the investigation of the motor's movement under a *constant* force. The current force signal (black arrow in Figure 1, right panel) is used to counterbalance the movement of the motor by moving the surface-bound microtubule in the opposite direction (blue arrow in Figure 1, right panel), reminiscent of a treadmill. In this case, the movement of the piezo-table records the movement of the motor as represented by the blue trace (Figure 1, right panel). Again, biophysical properties are readily extracted from these traces under constant load and saturating ATP conditions. The key biophysical parameters obtainable from these measurements are the *runlength* and the *velocity* of a motor, respectively. The slope represented in black dashed line reports on the velocity of a single motor. The double-headed arrow corresponds to the distance a single motor covers under a given force. This runlength is an important characteristic and reports on the degree of the motor's processivity, meaning how many consecutive steps it takes on its filament before it dissociates.

Most importantly, such a set-up allows us to resolve the discrete 'steps' of a single motor under limiting ATP conditions. Here the motor movement is sufficiently slow and reveals individual steps as illustrated by the zoom in Figure 1, right panel. Such traces deliver the most rigorous evidence for a motor's processivity and directly reports on the motor's *step size*. All processive kinesins characterized so far display an 8 nm step size.

Heterodimeric Kinesin-2: a unique representative of all double-head motors

One subclass of kinesins, kinesin-2, is unique in that some of its members are ***hetero***dimers combining two distinct subunits to one double-headed motor (Cole et al., 1993; Pan et al., 2006; Yamazaki et al., 1995). The C-terminus of the heterodimeric motor associates with a non–motor subunit, termed Kinesin Associated Protein (KAP) that is thought to link the motor to its cargo *in vivo* (Cole et al., 1993; Pan et al., 2006; Snow et al., 2004; Wedaman et al., 1996; Yamazaki et al., 1996). In *C.elegans* two member of the kinesin-2 class are at work to assemble and maintain the sensory cilia: the heterodimeric **K**inesin **L**ike **P**rotein 11 and 20 (KLP11/KLP20) and the *ho-mo*dimeric Osm-3 kinesin-2 (Figure 2). So far, these are the only known kinesins which co-operate to transport cargo in the *same* direction, namely to the cilia's tip (Ou et al., 2005; Snow et al., 2004).

Heterodimeric kinesin-2 apparently co-evolved with the cilia (Mitchell, 2004; Scholey, 2003; Silverman and Leroux, 2009). The question why nature combined two different polypeptide chains to create a heterodimeric motor specifically for ciliary function early in the evolution is intriguing and so far escaped our understanding. Below I will elaborate on how we can see through the nature's motivation to combine two different subunits to a double-headed motor by manipulating motors using advanced single molecule techniques.

Recurring kinetic signatures of long-range transporters

Processivity and auto-regulation are the two universal kinetic traits displayed by all kinesins involved in long-range intracellular transport. Auto-regulation is thought to be mediated by tail folding at the so-called hinge region. Such regulatory folding prevents futile ATP hydrolysis and is relieved when the tail domain of the motor binds to its cargo *in vivo* or *in vitro* (Dietrich et al., 2008; Espeut et al., 2008; Friedman and Vale, 1999; Hackney and Stock, 2000, 2008; Hammond et al., ; Hammond et al., 2009; Imanishi et al., 2006; Jiang and Sheetz, 1995). Cargo binding most likely causes a large conformational change in the molecule leading to a more extended conformation.

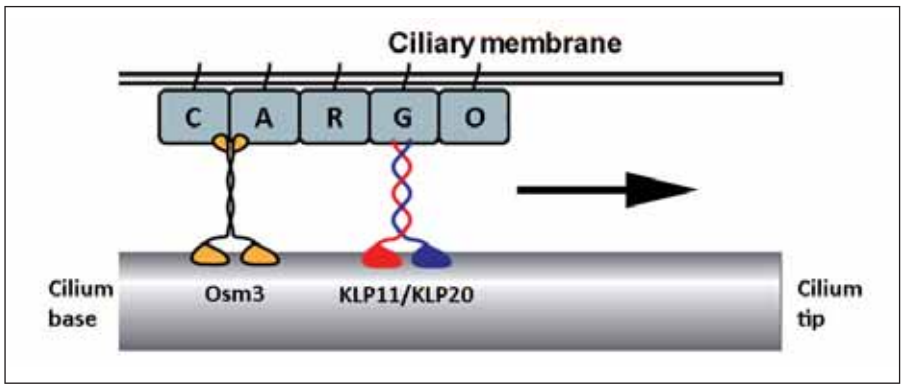

Figure 2: Illustration of the cargo transport in the C.elegans sensory cilium. The homodimeric Osm-3 and the heterodimeric KLP11/KLP20 kinesin-2 motors coordinate to enable the Intraflagellar Transport to the cilium's tip. The transport back to the cilium's base is accomplished by the dynein motor (not depicted).

Being involved in the long-range intraflagellar transport in the C.elegans sensory cilia, the homodimeric Osm-3 was accordingly shown to display both kinetic traits: Processivity and auto-regulation (Imanishi et al., 2006).

Identifying the kinetic signatures of a single KLP11/KLP20 heterodimer

As a single molecule, the heterodimeric KLP11/KLP20 displays 8-nm consecutive steps along the microtubule under limiting ATP conditions robustly underlining the motor's processivity (Figure 3, top panel). Noteworthy is the equivalence of the displayed steps by the two distinct motor subunits. If one subunit were a significantly faster motor than the other, one might have expected the motor to 'limp'. Such limping would become evident from alternating short and long dwells between steps which is not evident from the single molecule stepping traces.

The single molecule activity of the wild type motor measured under load and saturating ATP conditions, however, appears to be too infrequent, suggesting an auto-inhibited state (Figure 3, bottom panel, upper trace). Indeed, revoking the flexibility of the motor at the conserved hinge region by introducing rigid residues leads to a constitutively active motor as judged by the dramatically increased frequency of initiated runs (Figure 3, bottom panel). Importantly, this result can be reconstituted in bulk assays by measuring the motor's ATPase activity in solution.

The interrogation of the behavior of a single motor robustly identifies the key kinetic signatures of the wild type KLP11/KLP20: it is an auto-regulated and processive kinesin. Taken together these results now beg the question: Why is a heterodimeric motor needed at the first place? After all, Osm-3 kinesin achieves the same goal by being an ordinary homodimeric motor.

Dissecting the working mechanism of the heterodimeric KLP11/KLP20

The design concept of a heterodimeric motor appears more complicated when compared to its homodimeric counterparts. However, this complexity also provides unsurpassed opportunities to dissect the motor's working mechanism: Because of the two ***distinct*** identities, it is possible to pin-point the kinetic and structural contributions of the individual subunits to the intact motor protein (Brunnbauer et al.). Design of ap-

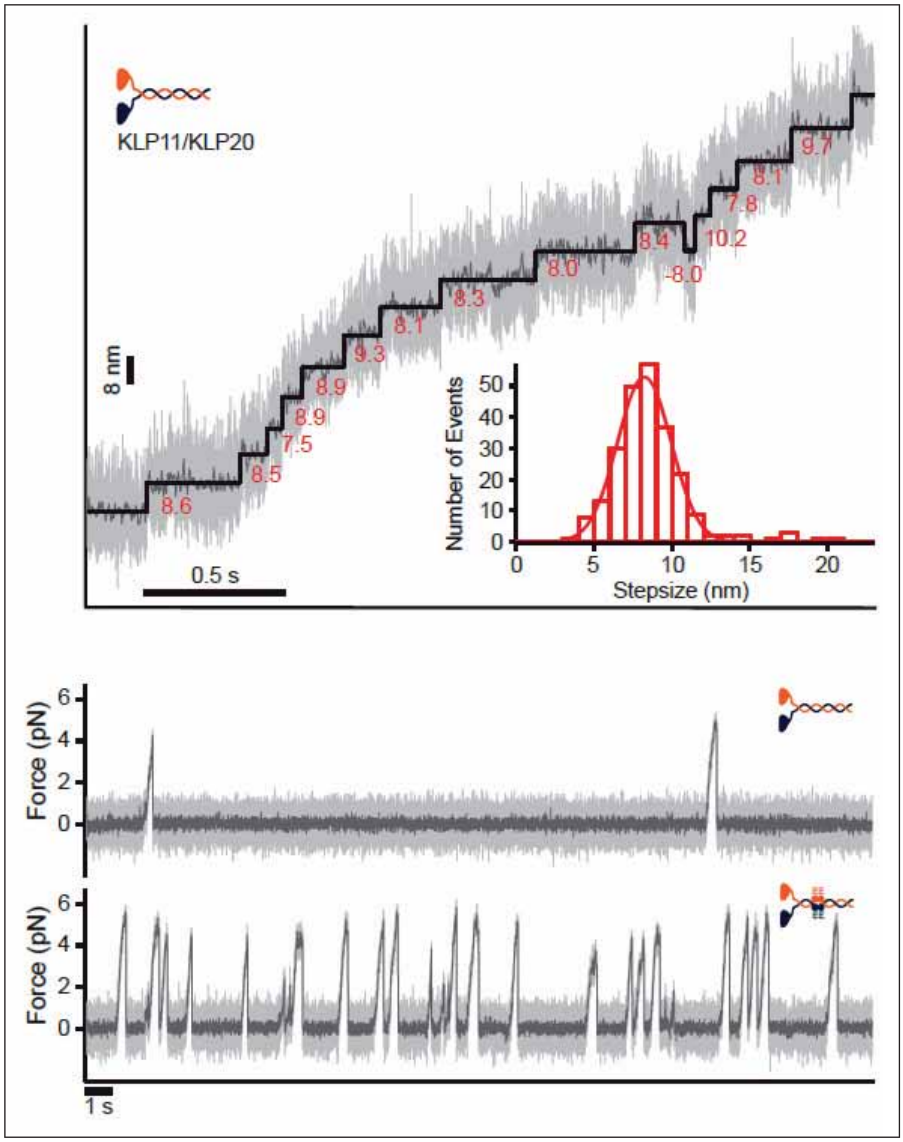

Figure 3: Single molecule behavior of the heterodimeric KLP11/KLP20 motor. Top panel shows the individual steps of a single KLP11/KLP20 molecule at subsaturating ATP and under constant load. The solid black line represents the steps as identified by an automated step-finder and red numbers give the step sizes of a single KLP11/KLP20 molecule. The inset shows the Gaussian fit to the histogram of step sizes revealing a step size of about 8 nm. Bottom panel illustrates how mutations in the tail domain of KLP11/KLP20 increase the frequency with which a single motor initiates a processive run. Here, the de-inhibition of the wild type motor is achieved by replacing of flexible residues in the hinge region by two glutamate residues (EE) per tail.

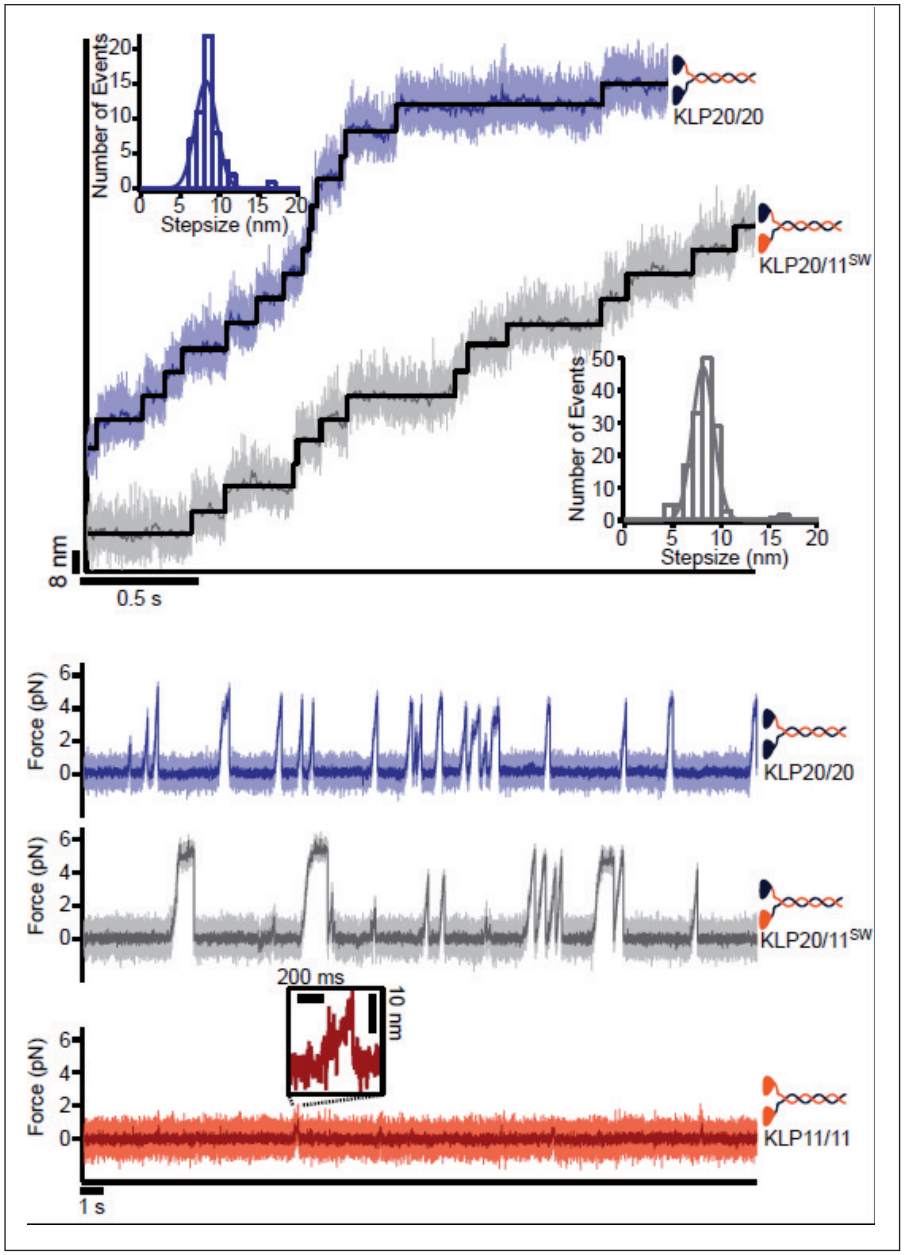

Figure 4: Single molecule behavior of the 'homodimeric' chimeras. Top panel illustrates the successful processive stepping of the KLP20/20 chimera (upper trace) and the double-chimeric KLP20/11^{sw} (lower trace) at subsaturating ATP revealing 8 nm step sizes (insets). Bottom panel illustrates the non-processive behavior of the KLP11/11 chimera. At saturating ATP single molecules of KLP20/20 (blue) and KLP20/11^{sw} (grey) initiate processive runs in stark contrast to the KLP11/11 chimera (red) which only generates rare 'twitches' in the detected signals meaning as a single molecule the combination of the KLP11 motor domains fails to take consecutive steps.

propriate chimeric constructs allows us to reduce the complexity to the level of a homodimer: excising one head domain in the heterodimer and replacing it with the other results in "homodimeric" chimeras (Figure 4). Here, the presence of the heterodimeric tail ensures proper dimerization. The double-chimeric construct with swapped heads is a vital control to ensure that the procedure of head transplantation does not give rise to deleterious effects.

The analysis of the stepping behavior of these chimeras now provides the first clues of why these subunits have to come together: the combination of the KLP20 motor domains to a "homodimeric " motor is capable of taking processive steps, as does the control chimera with swapped heads (Figure 4, top panel). However, the same is not true for the combination of the KLP11 head domains (Figure 4, bottom panel). This intriguing result underlines that for the wild type KLP11/KLP20 motor be processive, the KLP20 subunit must be present.

Why would nature retain the non-processive KLP subunit in a transport motor? The further analysis of the auto-regulation offers the clue. The motor activity of the "homodimeric" chimera KLP20/20 and the double-chimeric control with swapped heads were indistinguishable from the constitutively active KLP11/KLP20 motor (Figure 5, top panel). This analysis tells us that for the auto-regulation to take effect, the KLP11 motor domain must be present as the KLP20/20 is constitutively active. Remarkably, the sole presence of the KLP11 motor domain is not sufficient to confer an auto-regulated state either, as the control chimera with swapped heads is also de-regulated. Indeed, merely swapping the head domains leads to a constitutively active motor in ATPase assays in solution, independently confirming the conclusions from the single molecule experiments (Figure 5, bottom panel). In summary, the intact KLP11 subunit must be included in the wild type KLP11/KLP20 heterodimer to obtain an auto-regulated motor.

Taken together, the KLP11/KLP20 kinesin from C.elegans relies on a division of labor: While the KLP20 subunit is responsible for proper processivity, the non-processive KLP11 subunit is retained in the intact motor as is

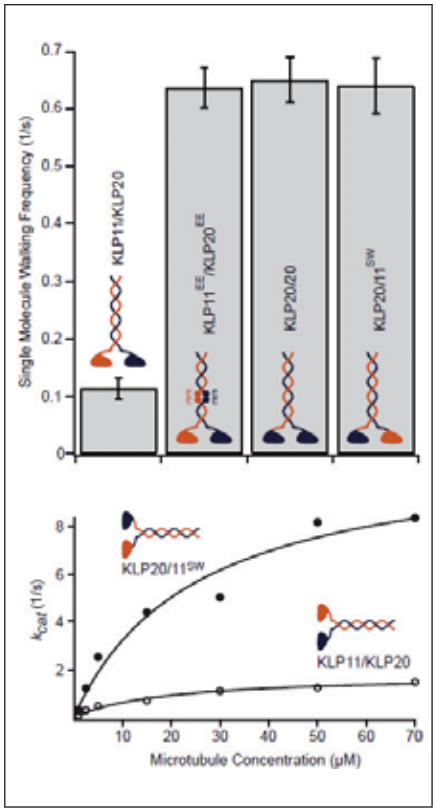

Figure 5: Dissecting autoinhibition of the constructs at single molecule level. Top panel shows that the autoinhibited wild type KLP11/KLP20 displays a walking frequency that is six-fold lower than the constitutively active motor containing the tail mutations. However, the walking frequency of the KLP20/20 and KLP20/11^{SW} chimeras both containing the *wild type tail* are quantitatively the same as that of the constitutively active motor, suggesting that autoinhibition is only possible if both KLP11 and KLP20 heads are present and are attached to their wild type tails. Bottom panel demonstrates that the suppressed ATPase of wild type KLP11/KLP20 heterodimer is relieved in the KLP20/11^{SW} chimera with swapped head domains independently validating our conclusions from the single molecule assays.

mediates the regulation of the heterodimer. The unmasking of such division of labor in the wild type KLP11/KLP20 heterodimer delivers the biological rational why nature has combined two distinct subunits in this particular kinesin-2 motor. With these conclusions I now would like to revisit our initial question: What can a single molecule teach us? As it turns out, more than one might have expected.

References

Brunnbauer, M., Mueller-Planitz, F., Kosem, S., Ho, T.H., Dombi, R., Gebhardt, J.C., Rief, M.Okten, Z., Regulation of a heterodimeric kinesin-2 through an unprocessive motor domain that is turned processive by its partner. *Proc Natl Acad Sci* U S A **107**, 10460-10465.

Cole, D.G., Chinn, S.W., Wedaman, K.P., Hall, K., Vuong, T.Scholey, J.M., 1993. Novel heterotrimeric kinesin-related protein purified from sea urchin eggs. *Nature* **366**, 268-270.

Dietrich, K.A., Sindelar, C.V., Brewer, P.D., Downing, K.H., Cremo, C.R.Rice, S.E., 2008. The kinesin-1 motor protein is regulated by a direct interaction of its head and tail. *Proc Natl Acad Sci* U S A **105**, 8938-8943.

Espeut, J., Gaussen, A., Bieling, P., Morin, V., Prieto, S., Fesquet, D., Surrey, T.Abrieu, A., 2008. Phosphorylation relieves autoinhibition of the kinetochore motor Cenp-E. *Mol Cell* **29**, 637-643.

Friedman, D.S.Vale, R.D., 1999. Single-molecule analysis of kinesin motility reveals regulation by the cargo-binding tail domain. *Nat Cell Biol* **1**, 293-297.

Hackney, D.D.Stock, M.F., 2000. Kinesin's IAK tail domain inhibits initial microtubule-stimulated ADP release. *Nat Cell Biol* **2**, 257-260.

Hackney, D.D.Stock, M.F., 2008. Kinesin tail domains and Mg2+ directly inhibit release of ADP from head domains in the absence of microtubules. *Biochemistry* **47**, 7770-7778.

Hammond, J.W., Blasius, T.L., Soppina, V., Cai, D.Verhey, K.J., Autoinhibition of the kinesin-2 motor KIF17 via dual intramolecular mechanisms. *J Cell Biol* **189**, 1013-1025.

Hammond, J.W., Cai, D., Blasius, T.L., Li, Z., Jiang, Y., Jih, G.T., Meyhofer, E.Verhey, K.J., 2009. Mammalian Kinesin-3 motors are dimeric in vivo and move by processive motility upon release of autoinhibition. *PLoS Biol* **7**, e72.

Hirokawa, N., Noda, Y., Tanaka, Y.Niwa, S., 2009. Kinesin superfamily motor proteins and intracellular transport. *Nat Rev Mol Cell Biol* **10**, 682-696.

Imanishi, M., Endres, N.F., Gennerich, A.Vale, R.D., 2006. Autoinhibition regulates the motility of the C. elegans intraflagellar transport motor OSM-3. *J Cell Biol* **174**, 931-937.

Jiang, M.Y.Sheetz, M.P., 1995. Cargo-activated ATPase activity of kinesin. Biophys J 68, 283S-284S; discussion 285S.

Mehta, A.D., Rock, R.S., Rief, M., Spudich, J.A., Mooseker, M.S.Cheney, R.E., 1999. Myosin-V is a processive actin-based motor. *Nature* **400**, 590-593.

Mitchell, D.R., 2004. Speculations on the evolution of 9+2 organelles and the role of central pair microtubules. *Biol Cell* **96**, 691-696.

Ou, G., Blacque, O.E., Snow, J.J., Leroux, M.R.Scholey, J.M., 2005. Functional coordination of intraflagellar transport motors. *Nature* **436**, 583-587.

Pan, X., Ou, G., Civelekoglu-Scholey, G., Blacque, O.E., Endres, N.F., Tao, L., Mogilner, A., Leroux, M.R., Vale, R.D.Scholey, J.M., 2006. Mechanism of transport of IFT particles in C. elegans cilia by the concerted action of kinesin-II and OSM-3 motors. *J Cell Biol* **174**, 1035-1045.

Scholey, J.M., 2003. Intraflagellar transport. *Annu Rev Cell Dev Biol* **19**, 423-443.

Silverman, M.A.Leroux, M.R., 2009. Intraflagellar transport and the generation of dynamic, structurally and functionally diverse cilia. *Trends Cell Biol* **19**, 306-316.

Snow, J.J., Ou, G., Gunnarson, A.L., Walker, M.R., Zhou, H.M., Brust-Mascher, I.Scholey, J.M., 2004. Two anterograde intraflagellar transport motors cooperate to build sensory cilia on C. elegans neurons. *Nat Cell Biol* **6**, 1109-1113.

Spudich, J.A., 1994. How molecular motors work. *Nature* **372**, 515-518.

Svoboda, K., Schmidt, C.F., Schnapp, B.J.Block, S.M., 1993. Direct observation of kinesin stepping by optical trapping interferometry. *Nature* **365**, 721-727.

Vale, R.D., 2003. The molecular motor toolbox for intracellular transport. *Cell* **112**, 467-480.

Wedaman, K.P., Meyer, D.W., Rashid, D.J., Cole, D.G.Scholey, J.M., 1996. Sequence and submolecular localization of the 115-kD accessory subunit of the heterotrimeric kinesin-II (KRP85/95) complex. *J Cell Biol* **132**, 371-380.

Yamazaki, H., Nakata, T., Okada, Y.Hirokawa, N., 1995. KIF3A/B: a heterodimeric kinesin superfamily protein that works as a microtubule plus end-directed motor for membrane organelle transport. *J Cell Biol* **130**, 1387-1399.

Yamazaki, H., Nakata, T., Okada, Y.Hirokawa, N., 1996. Cloning and characterization of KAP3: a novel kinesin superfamily-associated protein of KIF3A/3B. *Proc Natl Acad Sci* U S A **93**, 8443-8448.

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Break downs along the road

Thomas Eckert, Doan Tuong-Van Le, and Günther Woehlke

Background

Motoneurons control the action of muscles. In humans, most movements are steered by two levels of motoneurons organized in the so-called pyramidal system (Fig. 1). The first level of motoneurons (upper or first motoneurons) originates in the brain cortex and extends to the anterior horn in the spinal cord gray matter. There, the nerve pulses are transmitted to the second level, consisting of lower or secondary motoneurons.

There is a number of rare diseases causes by degeneration of central (first) and/or peripheral (secondary) motoneurons. Amyotrophic lateral sclerosis (ALS) is one example that is relatively well known to cell biologist because some cases are related to defects of the superoxide dismutase. It should be mentioned, though, that for most cases, the cause is unknown. The number and diversity of neurological disorders (WHO catalog International Classification of Diseases (ICD); http://apps.who.int/classifications/apps/icd/icd10online/) that are associated with the degeneration of motoneurons reflects the multitude of components involved in the maintenance of neurons, and suggests that neuronal survival mechanisms are tremendously complex. One of the Achilles heels of neuronal maintenance is the transport of organelles, proteins or other cargo.

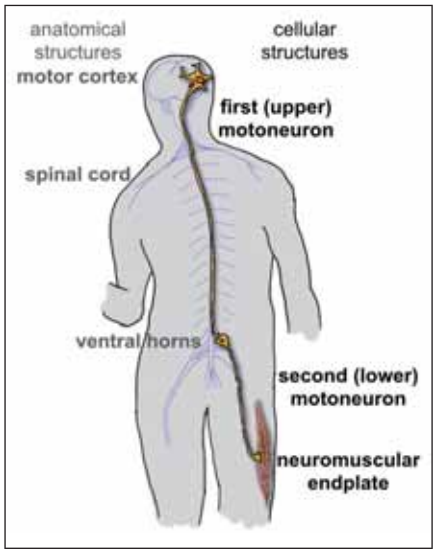

Figure 1: Schematic representation of the pyramidal motoric pathway. Hereditary spastic paraplegia mutations in KIF5A and spastin affect the integrity of the first motoneurons.

Motoneurons show a vivid bi-directional long-distance transport that depends on an intact microtubule cytoskeleton. Microtubule-dependent molecular motors are essential to carry a number of components along the axon into the direction, and from the direction of the synapse. If some of these motors, or the microtubule track are disturbed, the synapses structurally and functionally degenerate, and the nerve progressively looses its function. Thus, breakdowns can be caused by broken cars, or broken roads. This has been shown experimentally by introducing lesions to nerves, and in pathologic and genetically engineered cases where components of the transport system were defective. One neurological disorder that is relatively well studied is hereditary spastic paraplegia (HSP). Patients suffering from HSP develop a progressive spasticity and weakness of their lower limbs that is caused by the degeneration of upper motoneurons. A number of affected families has been studied, and several underlying genes have been mapped genetically (Reid, 2003). There are more than 40 known loci associated with HSP, and some of them contain genes that encode proteins related to the microtubule cytoskeleton. The most frequently encountered locus, SPG4, encodes spastin, an enzyme that severs microtubules at the expense of ATP and thus affects the dynamics of the microtubule cytoskeleton. Another locus, SPG10, contains the gene for conventional kinesin, isoform KIF5A. It is a molecular motor that moves cellular cargo towards the plus-ends of microtubules. In several unrelated families, point mutations in the catalytic domain of KIF5A have been correlated to the disease.

In this Research News article, we present our observations on the effect of HSP mutations in KIF5A and spastin in vitro. An important constraint for our studies is that patients with hereditary disorders often are heterozygous for the mutated gene. The crucial question is therefore, why patients develop the disorder although they still possess one intact copy of the gene. Two mechanisms can lead to the (genetically) dominant effect of the mutations: the mutated gene either leads to haplo-insuffiency, or interferes with the wildtype gene product such that it is unable to perform its normal function. Haploinsufficiency seems to occur frequently in reality (Beetz et al., 2006), but the point mutations we are studying show clear interferences with wildtype function and may reveal mechanisms of pathogenesis.

Effect of kinesin point mutations

Kinesin KIF5A is the major neuronal isoform of conventional kinesin in vertebrates. It is a processive microtubule motor that is thought to transport a number of cargos in the axon (Barry et al., 2007; Shah and Cleveland, 2002). The SPG10 locus comprises the KIF5A gene. Several missense mutations in parts encoding kinesin's N-terminal domains have been found to correlate with HSP (http://www.ncbi.nlm.nih.gov/omim/602821). Therefore, it is likely that kinesin transport defects cause the degene-

ration of motoneurons in affected persons.

The processive molecular motor kinesin uses the coordinated action of two identical motor domains to drive stepwise motility along the microtubule (Verbrugge et al., 2009; Woehlke and Schliwa, 2000). The fact that it consists of two (normally) identical subunits is important for HSP patients. As argued above, their genome contains two different alleles, one wildtype and one mutated allele. As most genes are expressed from both the maternal and the paternal chromosome, both the wildtype and the mutant polypeptide chains are present and able to form homo- and heterodimers. They will presumably form dimers randomly, leading to a mixture of 25% wildtype-wiltype homodimers, 50% wildtype-mutant heterodimers, and 25% mutant-mutant homodimers. We asked ourselves whether this mixture shows motility defects in vitro that might explain the occurrence of the disease (Ebbing et al., 2008). To this end, we used fluorescent quantum dots as cargo particles, mimicking the natural cargo. It is generally assumed that only few kinesin motors are attached to one cellular cargo. We therefore decorated the quantum dots with 3-5 molecular motors per particle. These motors were either all wildtype (as a control), or mixtures of wildtype and mutant in the stoichiometry 1:2:1 (wildtype-wildtype):(wildtype-mutant):(mutant-mutant). The run-length and the velocity histograms showed clear defects for the wildtype-mutant mixtures: While the wildtype-coated dots moved at ~ 0.8 µm/s and showed a single peak in the histogram, the mixtures were slower (two mutants) and showed a bi-modal distribution (one mutant; fig. 2). Moreover, two of the mutants caused a significant decrease of the landing rate on microtubules, and thus a lower frequency of processive runs per time and length of microtubule. These observations show clearly that the mutants are dominant in vitro. They presumably reduce the number of transported cargo per time, and/or slow down the gross velocity of the cargo particles.

There are some concerns about the transfer of our observations to the situation in vivo: It is known that kinesins are highly regulated. KIF5A seems to be involved in slow axonal transport, which takes place in the order of cm per week (Lasek, 1967; Xia et

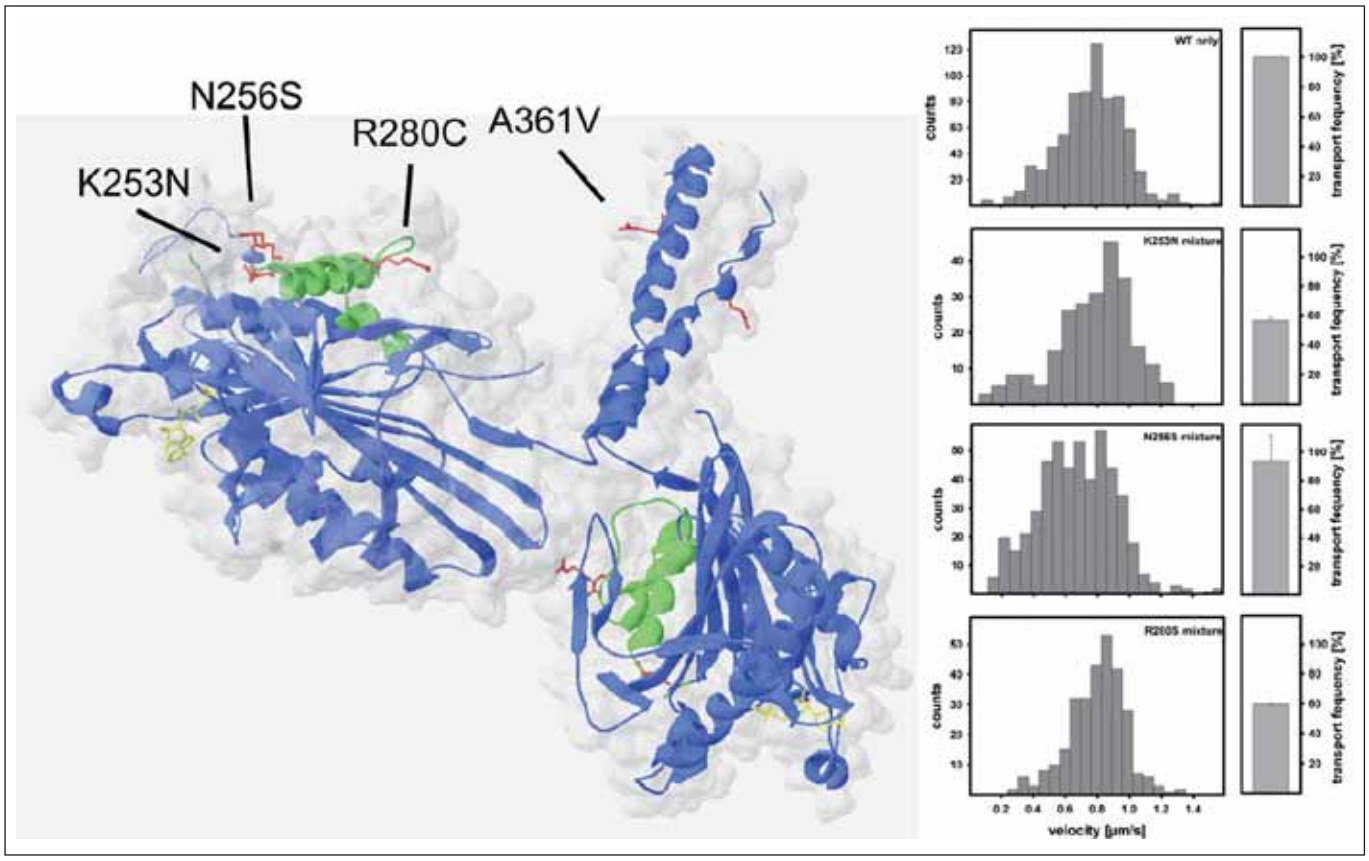

Figure 2: Model of kinesin's motor heads and location of HSP point mutations. Mixtures of homo- and heterodimeric wildtype / mutant motors were attached to quantum dot beads at the expected physiological ratio (see text), and added to microtubules that were fixed to a microscope coverslip. The movement of these coated beads along microtubules was recorded and analyzed for run-length, velocity and landing frequency. The histograms on the right show the velocities and landing frequencies (Ebbing et al., 2008).

al., 2003). In contrast, unregulated KIF5A (~1 µm/s velocity) would reach the end of a 1 m axon after 2 weeks, two orders of magnitude faster. Also, it is possible that KIF5A cargo particles also contain retrograde motors (cytoplasmic dynein), as KIF1A particles (Wang and Brown, 2010). It is therefore unclear whether the in vitro behavior can be directly extrapolated to the cellular environment. Future studies are necessary to solve the question of kinesin regulation.

Spastin and microtubule severing

Spastin was discovered as a cause for HSP (Hazan et al., 1999). The SPG4 gene turned out to encode an enzyme with close similarity to katanin, the first microtubule severing enzyme isolated from sea urchin sperm (McNally and Vale, 1993). The catalytic parts of both of these enzymes belong to the large family of AAA ATPases (ATPases associated with various cellular activities) that comprises enzymes as different as DNA helicases (SV40 large T antigen / SV40 helicase), protein unfoldases (ClpA/B), SNARE sorting enzymes (NSF), or enzymes that resolve ESCRT pathway scaffolds (Vps4). The unifying features of AAA ATPases are (i) sequence homology and conservation of characteristic motifs, (ii) activity as hexameric rings (only few members act as homo- or hetero-pentamers), and (iii) probably a substrate attack by mechanical means that is loosely coupled to ATP turnover. As their relatives, spastin (and katanin) are active as hexamers. The close similarity of spastin and other hexameric AAA ATPases, as well as a model based on small angle scattering that suggests a hexameric structure, strongly support this notion (Roll-Mecak and Vale, 2008; White et al., 2007).

As katanin, spastin possesses microtubule severing activity (Evans et al., 2005). Severing activity in the presence of severing enzyme and ATP is visible by eye in a microscopic assay in which microtubules are loosely fixed to the surface of a coverslip (Fig. 3). As microtubules are sensitive towards strong illumination, care has to be taken to perform the appropriate negative controls. Severing occurs rapidly after a few seconds lag phase in which the enzyme presumably assembles and accumulates to attack its microtubule-substrate. For spastin, a critical concentration is required. We estimate that the oligomerization of the hexameric form has a K_D in the µM range, and thus full activity requires this minimal enzyme concentration.

Severing is ATP-dependent. The ATP turnover can be measured in conventional coupled enzymatic assays and shows that spastin is a slow enzyme that consumes ~1 ATP / s per subunit in the absence, and ~4 ATP / s in the presence of microtubules. In comparison to kinesin, whose ATPase is activated by microtubules by 3 – 4 orders of magnitude, spastin shows only a minute activation. So far, it is unknown which step in spastin's kinetic cycle is accelerated by microtubules. It is an important future goal to elucidate the rate-limiting steps of spastin's reaction cycle and the shifts caused by microtubules, because these results will provide insight into the mechanism of action. As an alternative, we started the characterization of point mutants because we figured that they might arrest the severing reaction at defined intermediate states of the cycle. From a clinical point of view, these studies might indicate why certain alleles are dominant negative, and suggest strategies to circumvent the defects.

Two families with spastic paraplegia patients have been found to contain the same single-point nucleotide polymorphism in codon 499, leading to an Arg->Cys point mutation (Hazan et al., 1999; Svenson et al., 2001). In structure models, the absolutely residue Arg499 is located at the interface between two neighboring subunits. It reaches into the nucleotide-binding pocket of the neighbor subunit and might trigger ATP hydrolysis in a manner known for G proteins (Hillig et al., 1999; Ogura et al., 2004). Some G protein-activating proteins (GAPs) use "arginine fingers" to stabilize ATP hydrolysis intermediates. To prevent possible unspecific dimerization through the Arg499->Cys mutation, we studied an Arg499->Ala mutant (manuscript submitted). The mutant protein alone is completely inactive in severing and ATPase assays, highlighting the crucial role in catalysis (Fig. 4). For the clinical situation more relevant is a mixture of 50% wildtype and 50% mutant. In the presence of microtu-

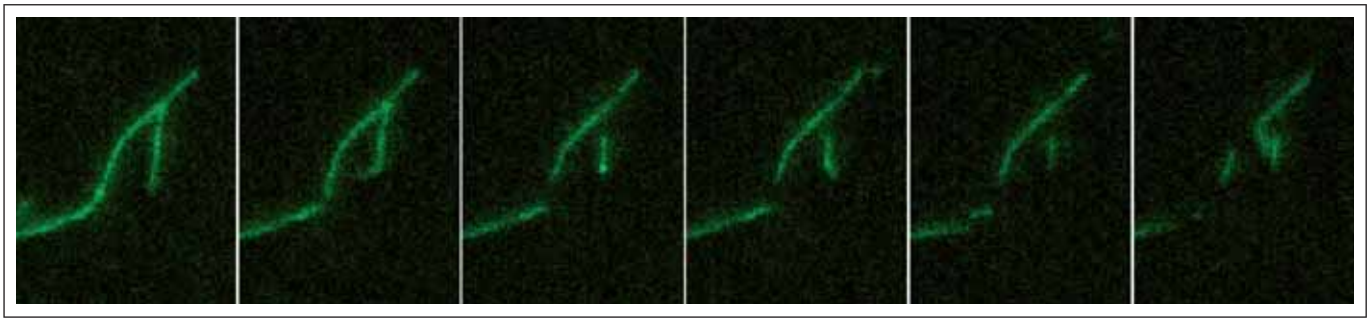

Figure 3: Severing activity of spastin. The picture shows a stack of images from a sequence of a few seconds. Microtubules are stained green and attached loosely to the coverslip. The filament breaks at several spots, yielding smaller fragments.

bules, the enzymatic activity is reduced from 4.0 ATP/s to ~ 1.6 ATP /s, a factor of 2.5. This is an amazing result because it shows that the "dead" mutant still interacts with wildtype. Although it is unable to hydrolyze ATP it apparently integrates into the hexameric ring. Otherwise, in our experimental setup with a constant amount of wildtype, the measured activity would be constant at all mutant concentrations. Remarkably, the shape of the inhibition curve is not linear: the activity at 50:50 wiltype:mutant is not the arithmetic mean of the activity of wildtype without any mutant, and the (extrapolated) activity of wildtype in hexamers where all wildtype subunits have a mutant neighbor. We attribute this to a higher stability of the wildtype-mutant neighborhood in comparison with a wildtype-wildtype neighborhood. Presumably, the wildtype-Arg499->Cys interface does not disassemble as quickly as wildtype-wildtype after completion of one round of ATP turnover. For the heterozygous patient, these data can explain the dominant effect of the Arg499->Cys mutation. Even a low background expression of the mutant variant will disturb the normal action of spastin, and hyper-stabilize axonal microtubules. Spastin has been found in the vicinity of centrosomes and in clusters along axons and dentrites, where they could be important for providing new, free plus-ends for polymerization, or short microtubules fragments that might be a substrate for transport to the synapse (Baas et al., 2005; Svenson et al., 2005). Thus, spastin mutations lead to non-functional transport roads.

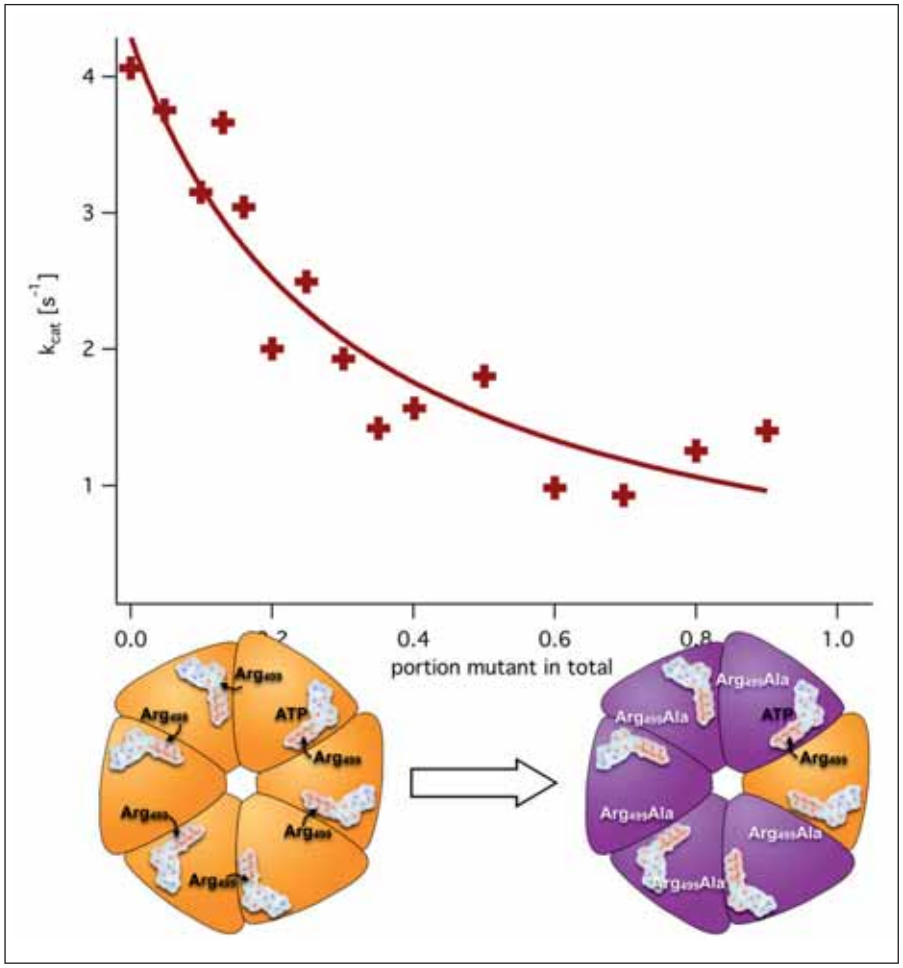

Figure 4: ATP turnover of spastin. The graph shows the ATPase activity of mixtures of wildtype and Arg499->Ala mutants at different mixing ratios. The wildtype concentration has been fixed, and variable amount of mutant have been added. A hyperbolic curve fit has been used to connect the data

Concluding Remarks

Why are mutations in components along the axonal transport road deleterious? The tips of motoneurons need a lot of energy supply, and one possibility is that KIF5A moves mitochondria to synapses. It has been observed that the depletion of mitochondria leads to the decay of intact synapses.

In addition, several other cargos are needed at the axon tip. Among them are neurofilaments, whose transport shows a clear connection to KIF5A. For example, KIF5A knockout mice show reduced transport of neurofilaments, and neurofilament motility is reduced in mouse neurons carrying HSP mutations (Wang and Brown, 2010; Xia et al., 2003). Axonal microtubules need to be dynamic, and for the nucleation of new microtubules, additional free plus-ends are required. This could be the task of spastin (Baas et al., 2005). Without the action of spastin, it would be impossible to regenerate microtubules that passed their natural lifetime. As any biological component, microtubules do not stay intact forever, but have a limited lifetime. Due to the peculiarities of the primate pyramidal system, it will be difficult (but not hopeless) to study HSP mutations in model organisms. Therefore, we will try to study the molecular defects "bottom-up", meaning that we will try to add more and more components and regulatory mechanisms to the mutated protein component. These studies are complex because they all have to be done with a wildtype protein background. Still, we think that this approach might complement the clinical studies and show whether the overexpression of wildtype components, or the

complementation with suppressors is a way to reduce the clinical symptoms.

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References

Baas, P.W., Karabay, A.Qiang, L., 2005. Microtubules cut and run. *Trends Cell Biol* **15**, 518-524.

Barry, D.M., Millecamps, S., Julien, J.P.Garcia, M.L., 2007. New movements in neurofilament transport, turnover and disease. *Exp Cell Res* **313**, 2110-2120.

Beetz, C., Nygren, A.O., Schickel, J., Auer-Grumbach, M., Burk, K., Heide, G., Kassubek, J., Klimpe, S., Klopstock, T., Kreuz, F., Otto, S., Schule, R., Schols, L., Sperfeld, A.D., Witte, O.W.Deufel, T., 2006. High frequency of partial SPAST deletions in autosomal dominant hereditary spastic paraplegia. *Neurology* **67**, 1926-1930.

Ebbing, B., Mann, K., Starosta, A., Jaud, J., Schols, L., Schule, R.Woehlke, G., 2008. Effect of spastic paraplegia mutations in KIF5A kinesin on transport activity. *Hum Mol Genet* **17**, 1245-1252.

Evans, K.J., Gomes, E.R., Reisenweber, S.M., Gundersen, G.G.Lauring, B.P., 2005. Linking axonal degeneration to microtubule remodeling by Spastin-mediated microtubule severing. *J Cell Biol* **168**, 599-606.

Hazan, J., Fonknechten, N., Mavel, D., Paternotte, C., Samson, D., Artiguenave, F., Davoine, C.-S., Cruaud, C., Durr, A., Wincker, P., Brottier, P., Cattolico, L., Barbe, V., Burgunder, J.-M., Prud'homme, J.-F., Brice, A., Fontaine, B., Heilig, R.Weissenbach, J., 1999. Spastin, a new AAA protein, is altered in the most frequent form of autosomal dominant spastic paraplegia. *Nat Genet* **23**, 296-303.

Hillig, R.C., Renault, L., Vetter, I.R., Drell, T.t., Wittinghofer, A.Becker, J., 1999. The crystal structure of rna1p: a new fold for a GTPase-activating protein. *Mol Cell* **3**, 781-791.

Lasek, R.J., 1967. Bidirectional transport of radioactively labelled axoplasmic components. *Nature* **216**, 1212-1214.

McNally, F.J.Vale, R.D., 1993. Identification of katanin, an ATPase that severs and disassembles stable microtubules. *Cell* **75**, 419-429.

Ogura, T., Whiteheart, S.W.Wilkinson, A.J., 2004. Conserved arginine residues implicated in ATP hydrolysis, nucleotide-sensing, and inter-subunit interactions in AAA and AAA+ ATPases. *Journal Of Structural Biology* **146**, 106-112.

Reid, E., 2003. Many pathways lead to hereditary spastic paraplegia. *Lancet Neurol* **2**, 210.

Roll-Mecak, A.Vale, R.D., 2008. Structural basis of microtubule severing by the hereditary spastic paraplegia protein spastin. *Nature* **451**, 363-367.

Shah, J.V.Cleveland, D.W., 2002. Slow axonal transport: fast motors in the slow lane. *Curr Opin Cell Biol* **14**, 58-62.

Svenson, I., Kloos, M., Jacon, A., Gallione, C., Horton, A., Pericak-Vance, M., Ehlers, M.Marchuk, D., 2005. Subcellular localization of spastin: implications for the pathogenesis of hereditary spastic paraplegia. *Neurogenetics* **6**, 135-141.

Svenson, I.K., Ashley-Koch, A.E., Gaskell, P.C., Riney, T.J., Cumming, W.J.K., Kingston, H.M., Hogan, E.L., Boustany, R.-M.N., Vance, J.M., Nance, M.A., Pericak-Vance, M.A.Marchuk, D.A., 2001. Identification and Expression Analysis of Spastin Gene Mutations in Hereditary Spastic Paraplegia. *The American Journal of Human Genetics* **68**, 1077-1085.

Verbrugge, S., Lechner, B., Woehlke, G.Peterman, E.J., 2009. Alternating-site mechanism of kinesin-1 characterized by single-molecule FRET using fluorescent ATP analogues. *Biophys J* **97**, 173-182.

Wang, L.Brown, A., 2010. A hereditary spastic paraplegia mutation in kinesin-1A/KIF5A disrupts neurofilament transport. *Mol Neurodegener* **5**, 52.

White, S.R., Evans, K.J., Lary, J., Cole, J.L.Lauring, B., 2007. Recognition of C-terminal amino acids in tubulin by pore loops in Spastin is important for microtubule severing. *J Cell Biol* **176**, 995-1005.

Woehlke, G.Schliwa, M., 2000. Walking on two heads: the many talents of kinesin. *Nat Rev Mol Cell Biol* **1**, 50-58.

Xia, C.H., Roberts, E.A., Her, L.S., Liu, X., Williams, D.S., Cleveland, D.W.Goldstein, L.S., 2003. Abnormal neurofilament transport caused by targeted disruption of neuronal kinesin heavy chain KIF5A. *J Cell Biol* **161**, 55-66.

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BRAF – the driving oncogene in pilocytic astrocytoma

Jan Gronych

Introduction

Gliomas are primary brain tumors encompassing different histologic subtypes which denote distinct biological and clinical behavior. Among those, pilocytic astrocytoma (PA) arise mainly in children, where they constitute the most common brain tumor[1]. Although after complete resection PA patients have an excellent prognosis, up to 20% of patients may not be cured by surgery alone since tumors grow in unfavourable anatomic locations where complete resection is impossible[2]. Due to the slow growing nature of PA, complete responses to radiation and chemotherapy are rare and most tumors eventually progress after a median stabilization period of typically more than 3 years[3]. Furthermore, adjuvant treatment results in severe adverse effects and neurologic deficits[4]. On the cellular level, recent studies suggested that the vast majority of PAs features constitutive activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling cascade as a molecular hallmark[5]. Molecular genetic analyses of primary tumor specimen have provided insights into the genetic alterations in these tumors including aberrations of the gene encoding for the BRAF proto-oncogene by duplication and subsequent gene fusion, or activating mutation[6-9]. All of these alterations were shown to confer constitutive kinase activity and elevated downstream signaling. While these molecular data are descriptive of the tumor characteristics, they did not provide functional evidence for the oncogenic relevance of BRAF in PA pathoge-

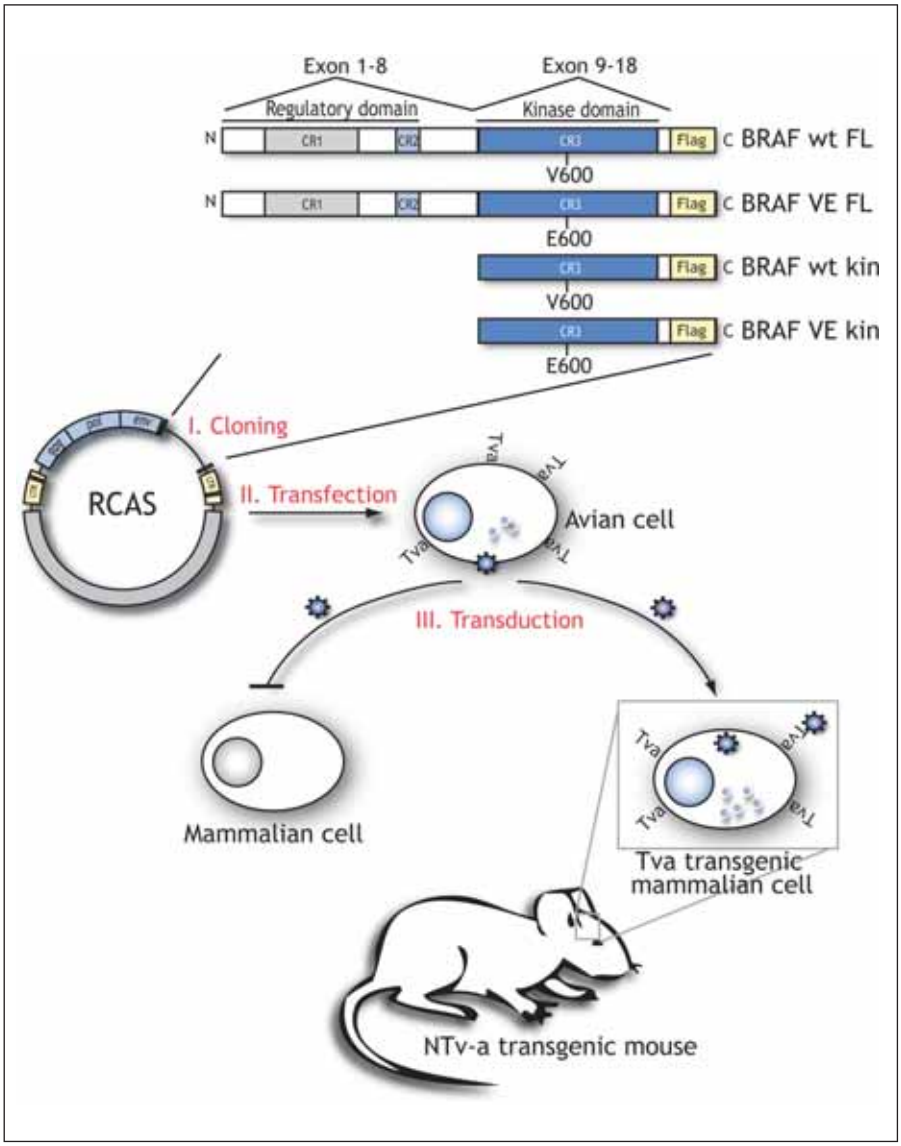

Figure 1: RCAS/Tva system for retroviral somatic gene transfer of different BRAF variants. BRAF variants have been generated and cloned into the RCAS retroviral plasmid. Viral plasmids are transfected into chicken fibroblasts which then allow efficient virus production. The generated RCAS virus is subsequently used for infection of mammalian cells transgenic for the Tva receptor, whereas normal mammalian cells are resistant to infection. In vivo infection is accomplished using NTv-a mice with transgenic Tv-a expression regulated by the nestin promoter.

nesis. In order to generate proof-of-principal data for the validation of BRAF as a potential drug target for therapeutic intervention, we applied *in vivo* somatic gene transfer for the expression of different BRAF variants in murine neural precursor cells *in situ*.

Molecular genetics of pilocytic astrocytoma

Over the past years, intensive work on the molecular genetic level using genomic and transcriptomic array-based methods has revealed a comprehensive picture of prevalent aberrations which contribute to the pathogenesis of glial tumors. At this point, these data are only descriptive for the disease´s molecular phenotype while causative relations need to be tested on a "candidate-by-candidate" basis in cell culture and/or animal models.

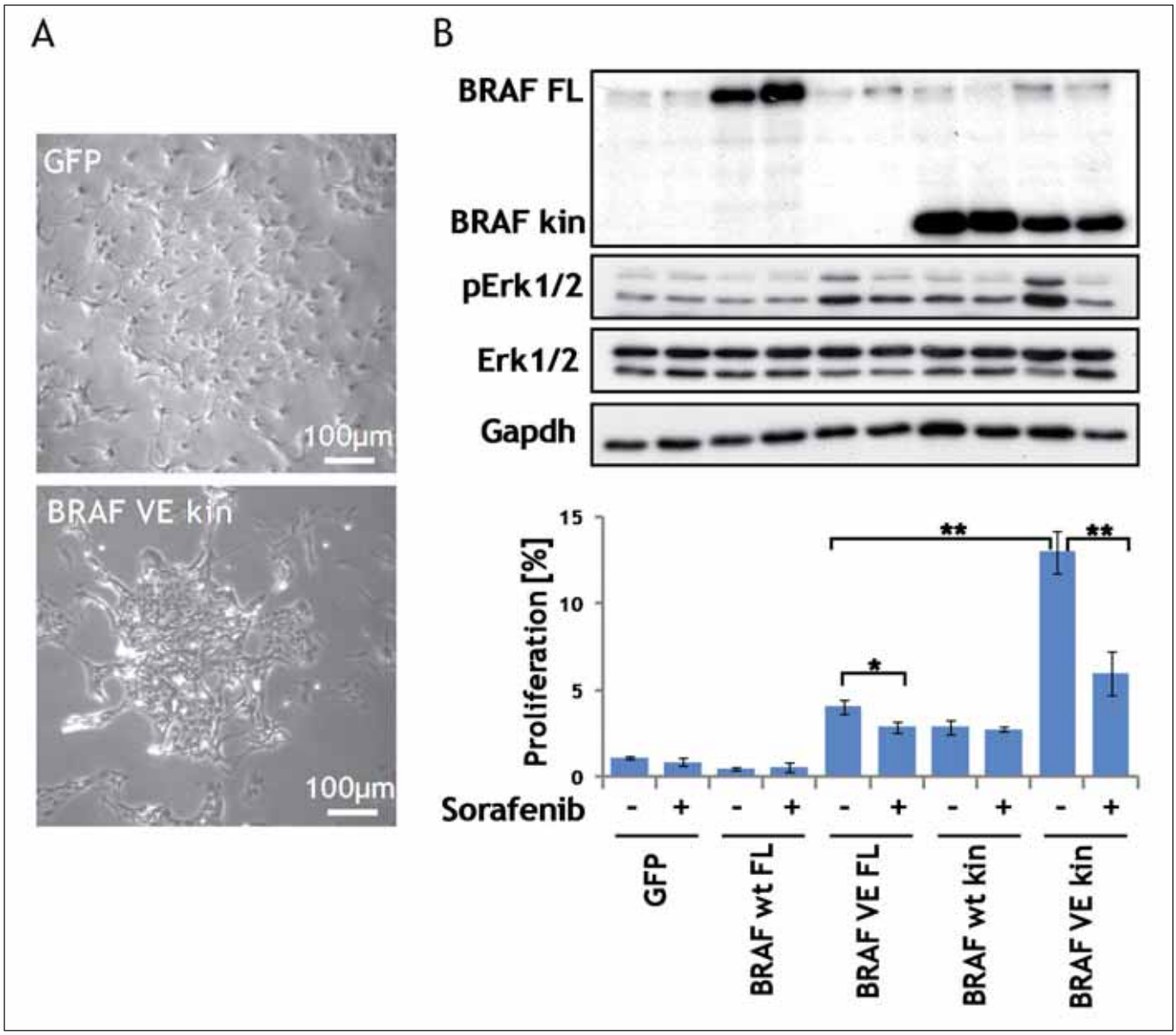

Figure 2: Expression of BRAF variants in primary NTv-a astrocytes. (A) Expression of BRAF VE kin alters cell morphology compared to GFP transduced control cells. (B) Western blot of cell lysates from NTv-a astrocytes transduced with BRAF constructs and treated with sorafenib as indicated. Detection with BRAF-specific antibody shows strong expression of all variants except for BRAF VE FL. BRAF VE kin induces strong Erk phosphorylation compared to the other variants and control. Treatment with sorafenib reduces Erk-phosphorylation to baseline levels Total Erk and Gapdh have been used as loading controls. Analysis of proliferation measured by EdU incorporation shows a strong proliferative effect of BRAF VE kin expression which can also be abrogated by sorafenib treatment.

For sporadic pilocytic astrocytoma, array-based comparative genomic hybridization (array-CGH) analysis revealed that these tumors widely display a balanced genomic profile with only harboring a focal duplication in chromosomal band 7q34 occurring at a high frequency[6,9]. The duplicated locus contains the BRAF proto-oncogene, a serine/threonine kinase component of the MAPK/ERK signaling cascade[10]. Subsequent studies showed that duplications result in the generation of fusion genes always encompassing exons 9-18, which encode for the BRAF kinase domain, and varying fusion partners, most commonly the yet uncharacterized gene *KIAA1549*[6,9,11,12] (Figure 1). In tumors harboring no BRAF gene fusion, an alternative aberration of this gene by a hotspot mutation at amino acid position 600 (Val → Glu, "V600E"), which had already been identified in melanoma, colorectal and thyroid carcinoma, or an in-frame insertion could be identified[9,13,14]. All these alterations result in a constitutive kinase activity of BRAF and enhanced MAPK signaling. These data suggested that MAPK activation plays an important role in PA pathogenesis. This was further supported by the fact that Neurofibromatosis patients suffering from a hereditary genetic disorder due to mutation of the gene NF-1 encoding for a negative regulator of Ras, frequently develop PAs[15].

Retroviral somatic gene transfer – RCAS/Tva technique

Mouse models have been widely used to investigate the *in vivo* function of genes with potential relevance in human diseases. In contrast to classical genetically engineered mouse models with germline transmission of the knock-in gene, somatic gene transfer using retroviral vectors offers a higher flexibility due to the less intricate implementation of new genes. This is further improved by the utilization of the *Replication Competent Avian leukosis virus with Splice acceptor* (RCAS), an avian viral vector[16]. It allows tissue specific infection in mice expressing the Tv-a transgene, the avian receptor essential for infection with RCAS, under control of a cell type specific promoter. Here, we used nestin promoter-driven Tv-a expression (NTv-a) resulting in infection of neural stem and progenitor cells in the mouse brain which has already been utilized previously for brain tumor models[17] (Figure 1). In addition, these progenitor cells can be cultivated under stem cell culture conditions for in vitro experiments. To investigate the role of different BRAF variants in neural progenitor cell proliferation and brain tumor pathogenesis, we used the RCAS/NTv-a system to ectopically express the BRAF wildtype full length (BRAF wt FL), the V600E mutated full-length variant (BRAF VE FL) and the truncated kinase domain corresponding to the BRAF portion of the fusion gene either wildtype (BRAF wt kin) or mutated (BRAF VE kin) *in vitro* and *in vivo* (Figure 1). Furthermore, we applied pharmacologic inhibition of BRAF with the small molecule sorafenib, which was shown to be a potent inhibitor of BRAF kinase activity.

BRAF induces proliferation of primary astrocytes in vitro

Primary astrocytes were transduced with the different RCAS-BRAF variants and assessed for transgene overexpression, MAPK activation (Erk phosphorylation) and proliferation. On the morphology level using brightfield microscopy, cells transduced with RCAS carrying *GFP* , *BRAF* wt FL, *BRAF* VE FL and *BRAF* wt kin revealed typical multipolar, astrocytic, flattened appearance and a growth pattern forming an expanded network of cells. Transduction with the mutated kinase domain (RCAS-*BRAF* VE kin), however, induced focal growth of the cells accompanied by bipolar/oligopolar morphology. Transgene expression was confirmed by real-time PCR (data not shown) and Western blot. All BRAF constructs were expressed on the RNA and protein level with the exception of BRAF VE FL, which showed a minimal protein expression despite considerable presence of the transcript (Figure 2 A). This is in line with earlier publications reporting decreased stability of the V600E mutated BRAF compared to the wildtype protein[18]. Erk phosphorylation indicative for MAPK activation was markedly increased in cells expressing BRAF VE kin, whereas there were low or minor effects seen in the other samples (Figure 2 A). Analysis of cell proliferation based on flow cytometric measurement of 5-ethynyl-2´-deoxyuridine (EdU) incorporation likewise revealed a strong proliferative effect of BRAF VE kin overexpression, while the other BRAF constructs induced no or only a low increase in proliferation (Figure 2 B). In both assays, pharmacologic inhibition of BRAF kinase activity strikingly reduced the effects caused by BRAF VE kin expression (Figure 2 A&B).

Murine tumor model

In order to investigate the tumorigenic potential of the different *BRAF* constructs, we infected neonatal NTv-a mice by intracranial injection. Thereby, neural progenitor/stem cells are transduced with the respective RCAS constructs leading to stable expression of the transgene. 17 weeks after RCAS-meditated delivery of the different *BRAF* variants all surviving mice were still clinically asymptomatic. Cranial magnetic resonance imaging (MRI) revealed a contrast enhancing region in T1-weighted images after application of contrast agent within the injected brain hemisphere in animals expressing BRAF VE kin (Figure 3 A). In the other groups, no such abnormality could be detected. Post mortem histopathologic analysis of dissected brains confirmed the presence of a neoplasia in the hyperintensive regions. Tumors were located either in one cerebral hemisphere, the brainstem or the cerebellum according to the respective site of injection. On the macroscopic level, tumors denoted strong expression of an astrocytic marker, the intermediate filament protein GFAP, and

diffuse transgene expression (Figure 3 A). Microscopic analysis of hematoxylin/eosin (H&E)-stained sections revealed moderate cellularity, fiber-rich tissue architecture including corkscrew-shaped eosinophilic structures, so called Rosenthal fibers, and tumor cells showing typical piloid morphology with elongated shape all reminiscent of human pilocytic astrocytoma. Also, tumors showed a very low proliferative index and a good demarcation from the surrounding healthy brain tissue, further characteristic features of these benign human neoplasias. Constitutive activity of MAPK signaling, which has already been shown in vitro, was also present in the RCAS-BRAF VE kin-induced tumors (Figure 3 B). These data indicate that the induced tumors resemble the human disease concerning the indolent growth and histologic characteristics. Furthermore, tumor induction with this approach was very efficient with an incidence of approximately 90% in animals injected with RCAS-*BRAF* VE kin.

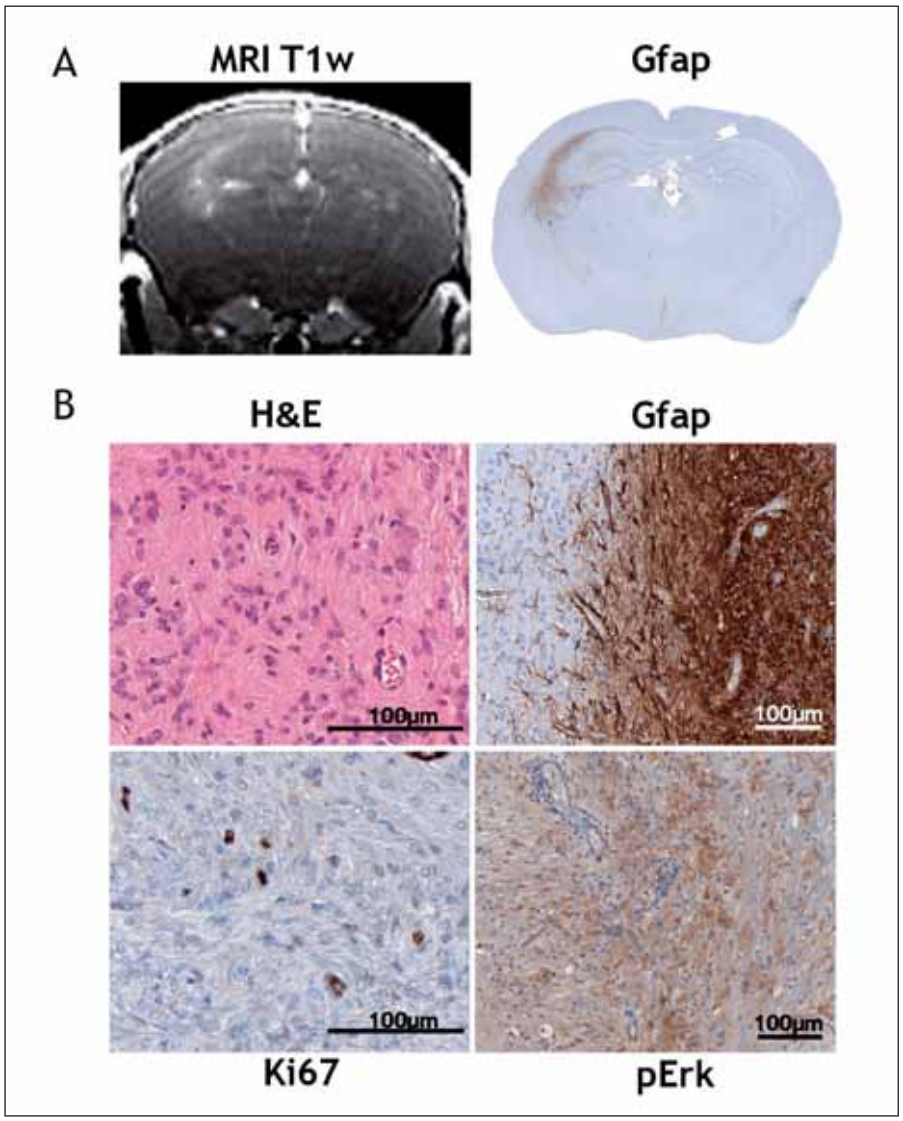

Figure 3: Induction of tumors in mice transduced with RCAS-BRAF VE kin. (A) 14 weeks after viral infection, unsymptomatic mice were subjected to MR imaging. T1-weighted (T1w) images after application of contrast agent reveals a hyperintensive region in the left hemisphere. Gfap staining on coronal sections shows an immunopositive lesion corresponding to the hyperintensive region. (B) Microscopic analysis of the induced tumors shows a fiber-rich tissue texture in H&E staining including Rosenthal fibers. Gfap Immunohistochemistry reveals a strong staining for this astrocyte marker in the non-invasive tumor and just a weak staining of reactive astrocytes in the surrounding normal tissue. Staining for the proliferation marker Ki67 is indicative for a low proliferation index. Immunohistochemical staining with a phosphor-specific Erk antibody illustrates the MAPK activation in the tumor.

Conclusion and outlook

Pilocytic astrocytoma constitutes a histologically benign childhood neoplasia of the brain. Analysis of genomic alterations of these tumors showed a high prevalence of aberrations of the *BRAF* gene that encodes a protein kinase of the MAPK signaling pathway and thus suggesting a role in PA tumorigenesis. Understanding the molecular pathomechanism of these tumors is an important prerequisite for the development of novel targeted therapies. However, appropriate model systems are essential for the preclinical evaluation of novel molecular targeted therapies. Due to the relatively benign nature of PA, no *in vivo* model and only a few cell culture models could be established for this neoplasm to date.

In order to investigate the impact of aberrant BRAF activation, we used retroviral somatic gene transfer for the ectopic expression of different BRAF variants *in vitro* using primary murine astrocytes as well as *in vivo* by intracranial injection. Ectopic expression of truncated BRAF harboring the V600E mutation lead to increased MAPK activation in murine astrocytes which was accompanied by increased proliferation and aberrant morphology. These effects could be abrogated by treatment of the cells with the kinase inhibitor sorafenib. Moreover, this construct was also able to induce tumors in mice which exhibited high similarity to tumors arising in children. Together, the data presented here

provide *in vivo* evidence for a causative role of constitutive BRAF activity for the pathogenesis of PA[19]. Furthermore, the established tumor model constitutes the basis for pre-clinical testing of novel therapy options which should pave the way to targeted therapy options in clinical trials.

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References

1. World Health Organization & International Agency for Research on Cancer. *WHO classification of tumours of the central nervous system*, (International Agency for Research on Cancer, Lyon, 2007).
2. Dirven, C.M., Mooij, J.J. & Molenaar, W.M. Cerebellar pilocytic astrocytoma: a treatment protocol based upon analysis of 73 cases and a review of the literature. *Childs Nerv Syst* **13**, 17-23 (1997).
3. Pfister, S. & Witt, O. Pediatric gliomas. *Recent Results Cancer Res.* **171**, 67-81 (2009).
4. Mulhern, R.K., Merchant, T.E., Gajjar, A., Reddick, W.E. & Kun, L.E. Late neurocognitive sequelae in survivors of brain tumours in childhood. *Lancet Oncol* **5**, 399-408 (2004).
5. Sharma, M.K., Zehnbauer, B.A., Watson, M.A. & Gutmann, D.H. RAS pathway activation and an oncogenic RAS mutation in sporadic pilocytic astrocytoma. *Neurology* **65**, 1335-1336 (2005).
6. Pfister, S., et al. BRAF gene duplication constitutes a mechanism of MAPK pathway activation in low-grade astrocytomas. *J Clin Invest* (2008).
7. Forshew, T., et al. Activation of the ERK/MAPK pathway: a signature genetic defect in posterior fossa pilocytic astrocytomas. *J Pathol* **218**, 172-181 (2009).
8. Jones, D.T.W., et al. Oncogenic RAF1 rearrangement and a novel BRAF mutation as alternatives to KIAA1549:BRAF fusion in activating the MAPK pathway in pilocytic astrocytoma. *Oncogene* **28**, 2119-2123 (2009).
9. Sievert, A.J., et al. Duplication of 7q34 in pediatric low-grade astrocytomas detected by high-density single-nucleotide polymorphism-based genotype arrays results in a novel BRAF fusion gene. *Brain Pathol* **19**, 449-458 (2009).
10. Robinson, M.J. & Cobb, M.H. Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* **9**, 180-186 (1997).
11. Jones, D.T.W., et al. Tandem Duplication Producing a Novel Oncogenic BRAF Fusion Gene Defines the Majority of Pilocytic Astrocytomas. *Cancer Res* **68**, 8673-8677 (2008).
12. Cin, H., et al. Oncogenic FAM131B-BRAF Fusion Gene Resulting from 7q34 Deletion Comprises an Alternative Mechanism of MAPK Pathway Activation in Pilocytic Astrocytoma. *Acta Neuropathol* In Press(2011). 2011 Jun; 121(6):763-74.
13. Eisenhardt, A.E., et al. Functional characterization of a BRAF insertion mutant associated with pilocytic astrocytoma. *Int J Cancer* (2010).
14. Schindler, G., et al. Analysis of BRAF V600E mutation in 1,320 nervous system tumors reveals high mutation frequencies in pleomorphic xanthoastrocytoma, ganglioglioma and extra-cerebellar pilocytic astrocytoma. *Acta Neuropathol* **121**, 397-405 (2011).
15. Listernick, R., Charrow, J. & Gutmann, D.H. Intracranial gliomas in neurofibromatosis type 1. *Am J Med Genet* **89**, 38-44 (1999).
16. Hughes, S.H. The RCAS vector system. *Folia Biol (Praha)* **50**, 107-119 (2004).
17. Dai, C., et al. PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo. *Genes Dev* **15**, 1913-1925 (2001).
18. Grbovic, O.M., et al. V600E B-Raf requires the Hsp90 chaperone for stability and is degraded in response to Hsp90 inhibitors. *Proc Natl Acad Sci U S A* **103**, 57-62 (2006).
19. Gronych, J., et al. An activated mutant BRAF kinase domain is sufficient to induce pilocytic astrocytoma in mice. *J Clin Invest* **121**, 1344-1348 (2011).

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The study section "Cell Biology of viral infections" of the German Society of Virology (GfV) invites interested cell biologists to participate in our annual meeting held in **Deidesheim, September 21 – 23, 2011.** This meeting is aimed to bring cell biologists and virologists together and foster collaborations and understanding for each others approaches.

The Background

More then 100 years ago all that was known about viruses was their ability to retain their infectious potential after filtration through membranes that effectively removed cells and bacteria. It required the development of electron microscopy to identify their particulate nature showing the importance of technological advancement. The concept that viruses are infectious particles opened the way to study host-pathogen interactions as we still do today.

However, the study of viruses as disease causing agents is only one part of the story. The investigation of virus biology and their interaction with cells led to the identification of several fundamental principles of cell biology and their impact on human health. Indeed the ability of viruses to cause cell proliferation or transformation (cancer) was known long before the first virus was spotted in a microscope (summarized in an excellent recent review by Kalland et al. 2009). Subsequent research using DNA tumor viruses and retroviruses showed how viral proteins interact, modify and ultimately subvert cellular proteins thus regulating cell cycle, block apoptosis and induce proliferation.

But there is more. Transcriptional activation and RNA processing like polyadenylation (Kates and Beeson 1970), capping (Furuichi et al. 1975), splicing (Berget et al. 1977; Chow et al. 2000) and editing (Cattaneo et al. 1989) have all been discovered using viral model systems (Kalland et al. 2009). The dogma of the unidirectional gene expression DNA-RNA-protein has been challenged by the discovery of the reverse transcriptase (hence their name !) in retroviruses (Baltimore 1970). As such viruses as tools in cell biology received their fair share of nobel prizes.

Analysis of the SV40 virus large T antigen, a nuclear resident, allowed the identification of small lysine-arginine-rich peptides as basis of its nuclear import leading to the discovery of nuclear localization sequences (NLS, (Kalderon et al. 1984)). Another viral protein, the Rev protein of HIV helped to identify nuclear export signals (NES, (Kalland et al. 1994; Meyer and Malim 1994)). Subsequently the SV40 T-antigen and Rev protein provided an experimental system to characterize import and export receptors and the regulation of nucleocytoplasmic transport.

From the above it becomes clear that viruses are excellent tools to study cell biological processes due to their need to use and modify the cellular system to fulfil their own infectious replication cycle. Often this involves the induction of proliferation in otherwise terminally differentiated cells by activating metabolic activity and cell cycle progression to support viral growth. At the same time the virus needs to limit cellular antiviral defence mechanisms or prevent cells from undergoing apoptosis while maximally exploiting the cellular machinery. On the whole this is achieved with very few viral proteins although lately it has become clear that viral nucleic acids (e.g. miRNAs) can substantially contribute to viral control of the cell.

Viruses are also exquisite transport substrates. (Some) viruses have to reach the nucleus for replication. To do so they have to cross membranes, engage in cytosolic transport followed by nuclear import and intranuclear transport. Following replication and (nuclear) assembly of new viruses this transport is often reversed for virus release at the cell surface using apparently the same set of proteins. Many aspects of directionality of virus transport (and as a consequence the directionality of cellular transport) remain enigmatic even today.

By nature a virus is limited in its coding capacity to perform all the above functions. Thus viral factors have to be efficient. Efficiency comes from interfering with key cellular pathways. One way of achieving this is by providing high affinity binding sites that simply outcompete cellular factors. Alternatively, cellular factors may be destroyed or mislocalized leading to orphaned physiological binding partners and hijacking of pathways. Viruses may accomplish that by post-translational modification of cellular factors. Indeed several viruses encode kinases, proteases or ubiquitin ligases that alter the specificity and/or the stability of cellular proteins.

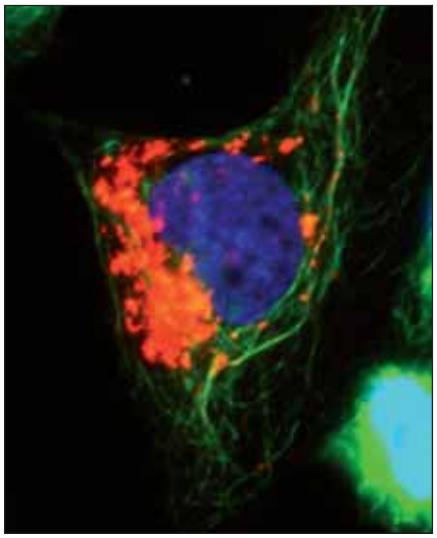

Figure 1: The capsid protein VI of Ad associates with microtubules: Expressed adenoviral capsid protein VI fused to mRFP (red) localizes into dynamic vesicular-tubular structures that move along microtubules (green) in a PPxY dependent manner (Wodrich et al. 2010).

In summary viral proteins are valuable tools to direct attention to cellular pathways and important physiological interactions. The purpose of our meeting is to bring scientists from the virus and the cellular world together to share their observations.

The Organizers

Harald Wodrich: From early on, my dream combination for research has been Cell biology and Virology. During my PhD I worked in the group of Hans-Georg Kräusslich at the Heinrich-Pette-Institute in Hamburg on nuclear export and splicing of retroviral RNAs. These RNAs are unique in that they can be exported even if (some) introns remain unspliced, something that the cell does not tolerate. The addition of short sequence elements termed post-transcriptional regulatory elements (e.g. the Rev-RRE from HIV, CTE from Mason Pfizer monkey Virus, PRE from HBV) overcomes this restriction via high-affinity binding sites for cellular export factors (e.g. Tap for the CTE). We subsequently generated gene transfer vectors or DNA based vaccines with increased expression properties solely by adding one or several of these short sequence elements providing evidence that understanding fundamental cell biological processes could be applied to create new tools (Wodrich and Krausslich 2001).

For my postdoc I tried to leave the virus field because I became more interested in cell biology. I was offered a unique project to investigate nuclear import of large macromolecules by Larry Gerace at the Scripps Research Institute in La Jolla, USA. Needless to say that the proposed model system for the macromolecule was the adenoviral genome, once again an excellent example where viruses serve as tools to address fundamental cell biological questions. Our work suggested that nuclear import of the viral genome uses the histone-like viral protein VII, which coats the genome in regular intervals. This protein provides the necessary NLS to link the genome to the cellular protein import machinery (Wodrich et al. 2006). Different work showed that cleavage of nucleocytoplasmic transport signals in the capsid protein VI, another adenoviral factor, could trigger virus assembly showing conversion of a transport-adapter into a structural protein (Wodrich et al. 2003). This protein also encoded an amphipathic helix for membrane interaction and a PPxY ubiquitin ligase binding motif. The identification of an amphipathic helix in the internal capsid protein of a non-enveloped virus was puzzling at first. Studies carried out with the group of Glen Nemerow at Scripps showed that indeed this protein was responsible for the lysis of endosomal membranes and allows adenoviruses to enter the cytoplasm after endocytic uptake adding yet another function to the same protein (Wiethoff et al. 2005).

At my next career stop I returned to Europe, to France to work as a senior scientist at the Institute for molecular Genetics in Montpellier. Montpellier is a French hotspot for Cell Biology with major groups working in cell cycle regulation and post-translational modification (e.g. ubiquitin & SUMO). Here I further characterized the PPxY motif in protein VI. Working with a viral protein again proved to be ideal to investigate cellular pathways. The PPxY showed to be essential for intracellular transport related to virus infectivity (Fig. 1, Wodrich et al. 2010). Since 2008 my group is at the University of Bordeaux on the French Atlantic coast. Our work focuses on three topics, 1) the physiological role of post-translational modifications of adenoviruses during virus entry, 2) the pathophysiological consequences of these modifications for the host, and lastly 3) the regulation of virus induced cell-proliferation and transcriptional activation. Although these all seem like virological questions reality shows that what we study is 1) the mechanism of post-translational modification in cells, 2) alterations in intracellular transport as well as 3) cell signalling and cell cycle control. As such I am convinced that Cell biology and Virology adress similar questions merely looking from different angles.

Susanne M. Bailer: Unlike Harald Wodrich, I started my scientific life in a cell biological environment. My interest in functions and dynamics of the nuclear envelope and the nuclear pore complex (NPC) was sparked in the lab of Erich A. Nigg at the Institute of Cell Biology, ETH Zürich, where I received my PhD. It was further deepened during postdoctoral research at the National Institutes of Health/ Bethesda and in particular in Ed Hurt's lab at the Biochemiezentrum Heidelberg where I contributed to unravel the organisation and function of the S. cerevisiae NPC (Bailer et al. 2001).

With my own lab at the University of the Saarland, Homburg/Saar, I continued to work on nucleo-cytoplasmic transport in S. cerevisiae (Betz et al. 2004; Fries et al. 2007) while establishing Herpes simplex virus 1 (HSV-1), a prototype herpesvirus, as a novel model system. Amenable to genetic, biochemical and cell-biological approaches HSV-1 offers experimental opportunities similar to yeast however in higher eukaryotic cells. In particular, since a considerable part of the herpesviral life cycle takes place in the host nucleus HSV-1 is an ideal tracer of chromatin and nuclear envelope related cellular processes.

Formation of HSV-1 infectious particles starts in the nucleus followed by cytoplasmic maturation and envelopment at the trans-Golgi network. Exceeding the size limit of the pore diameter, nuclear capsids reach the cytoplasm by budding through the nuclear envelope. "Nuclear egress", a subject of great interest in herpesvirology, relies on UL34p, an integral membrane protein that interacts with UL31p to form the nuclear egress complex (NEC) at the inner nuclear membrane (INM; Fig. 2). Recently we showed that UL34p contains a tail-anchor (TA) domain for posttranslational insertion into target membranes (Ott et al., in revision). Using the HSV-1 bacterial artificial chromosome (BAC) we defined features of UL34 essential for membrane insertion

and viral function that gave first mechanistic insight into the particular role of its TA in membrane curving and capsid egress from the nucleus. Current efforts focus on the analysis of INM targeting of UL34p as well as glycoprotein M (gM), a type III integral membrane protein that likely assists in nuclear egress. Interestingly, gM resides in various membrane compartments depending on the stage of infection suggesting its distribution is regulated. Together our analyses demonstrate the potential of HSV-1 as a genetic system to feed two fields of high interest in cell biology, namely targeting of integral membrane proteins to the INM as well as TA protein biogenesis.

Nuclear morphogenesis of capsids starts at discrete foci, which grow in size eventually claiming considerable space of the nucleus and marginalizing the host chromatin. Ongoing viral propagation is thus accompanied by massive alterations of the host chromatin. How these alterations are brought about is largely unknown. Based on protein interaction screens and RNAi we have identified several chromatin-associated proteins that functionally impact viral propagation and at the same time physically interact with viral candidates (Schmidt et al. 2010). Our work aims at deciphering the molecular details of virus-host chromatin interaction that also is an important aspect of nuclear egress.

Finally, since nucleo-cytoplasmic exchange of viral proteins is vital for productive infection it naturally triggered my interest. Its comprehensive analysis was facilitated by an HSV-1 Orfeome-wide gene collection (Fossum et al. 2009). In the Haas/Koszinowski lab at the Max von Pettenkofer-Institut, LMU München, I developed a novel inducible and sequence independent nuclear export assay to overcome experimental limitations notoriously associated with analysis of nuclear export. Surprisingly, systematic analysis of HSV-1 protein export unveiled unknown export activities that point to novel regulatory principles of infection. Remarkably this brings me back to the point where I started my career showing the intricate connection of Virology and Cell biology.

The Meeting

It is obvious that we continue to be fascinated by the variety of cell biological processes that one can study and understand by looking at the viral life cycle. Promoting this great combination is one of our major goals as speakers of the study section "Cell Biology of viral infections". The workshop we are organizing was founded in 2001 at Zeilitzheim and initially organized by Beate Sodeik and Michael Kann; and later continued in Deidesheim, first by Stefan Urban and Jacomine Krijnse-Locker and followed by Kay Grünewald and Mario Schelhaas. Its aim is to bring cell biologists and virologists closer together and to enhance exchange and discussions between these fields.

This year the workshop is focused on "Modification of gene expression". In good tradition the meeting will be flanked by four keynote speakers: **Gunter Meister** started his work on small noncoding RNAs, their processing and associated proteins in the Tuschl lab at The Rockefeller University, New York, and gained early independence as a junior group leader at the Max Planck Institute for Biochemistry, Martinsried/Munich. Since 2009, he is head of the department of biochemistry at the University of Regensburg. **Stefan Hüttelmaier** was a postdoctoral fellow of RH Singer at the Albert Einstein College of Medicine, New York, where the zipcode driven spatio-temporal distribution of mRNAs was pioneered. Back in Germany, Stefan Hüttelmaier established an NBL3 junior research group prior to becoming professor of Molecular Cell Biology and director of the Core Facility Imaging at the Martin-Luther-University Halle-Wittenberg. Using imaging technologies, Stefan Hüttelmaier is particularly interested in translational control of mRNAs by RNA binding proteins and miRNAs, which determine their distribution and fate. The research by **Carsten Janke** is specialized on the post-translational modification of microtubules. After his postdoc he was recruited as a CNRS senior researcher to the laboratory of Bernard Eddé where he identified the first tubulin glutamylase. He started his own group in Montpellier followed by an appointment as group leader at the Institut Curie, Paris. His work led to the identification of a new protein family dedicated to posttranslational modifications of tubulin, including glutamylation, which are important for tubulin function. Recently glutamylation was shown to be a widespread phenomenon particularly abundant on nucleo-cytoplasmic shuttling and chromatin-binding proteins. **Thomas Sternsdorf**, an alumni of the Salk Institute for Biological Studies, La Jolla, is currently based at the Forschungsinstitut Kinderkrebs-Zentrum Hamburg. A holder of a Heisenberg scholarship, Thomas Sternsdorf is working on leukemias and analysing sumoylation, another posttranslational modification of proteins. The functional consequence of conjugating proteins to SUMO (Small Ubiquitin-like MOdi-

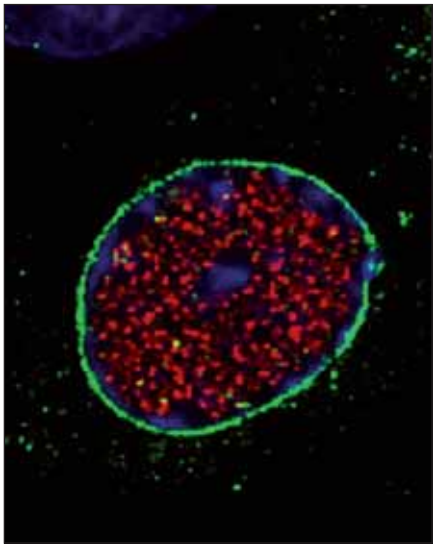

Figure 2: HSV-1 capsids exit the nucleus by membrane budding. Capsids formed in intranuclear replication compartments upon HSV-1 infection (red, anti ICP8 antibodies provided by R. Heilbronn, Charité) leave the nucleus by budding through the inner nuclear membrane where the NEC protein UL34 (green) acts as receptor (Courtesy of M. Ott, LMU München).

fier), a protein that structurally resembles ubiquitin, is still under investigation, however its dynamic and reversible nature suggests its potential to regulate target protein behavior.

This year´s workshop will again take place at the Ketschauer Hof in Deidesheim, located in the beautiful surroundings of the Pfalz. Researchers of the Cell biology and Virology fields at all levels of their carreer are invited to provide fascinating contributions, to join us in lively discussions and to celebrate the 10th anniversary of this exceptional workshop.

Details for the workshop can be obtained at the website of the GfV (http://www.g-f-v.org/) or the workshop (http://mcmp.aquitaine.cnrs.fr/mfp/viromeeting/announcement.php), or by directly contacting the organizers.

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References:

Bailer SM, Balduf C, Hurt E (2001) The Nsp1p carboxy-terminal domain is organized into functionally distinct coiled-coil regions required for assembly of nucleoporin subcomplexes and nucleocytoplasmic transport. *Mol Cell Biol* **21**(23): 7944-7955.

Baltimore D (1970) RNA-dependent DNA polymerase in virions of RNA tumour viruses. *Nature* **226**(5252): 1209-1211.

Berget SM, Moore C, Sharp PA (1977) Spliced segments at the 5' terminus of adenovirus 2 late mRNA. *Proc Natl Acad Sci U S A* **74**(8): 3171-3175.

Betz C, Schlenstedt G, Bailer SM (2004) Asr1p, a novel yeast ring/PHD finger protein, signals alcohol stress to the nucleus. *J Biol Chem* **279**(27): 28174-28181.

Cattaneo R, Kaelin K, Baczko K, Billeter MA (1989) Measles virus editing provides an additional cysteine-rich protein. *Cell* **56**(5): 759-764.

Chow LC, Gelinas RE, Broker TR, Roberts RJ (2000) An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. 1977. *Rev Med Virol* **10**(6): 362-371; discussion 355-366.

Fossum E, Friedel CC, Rajagopala SV, Titz B, Baiker A et al. (2009) Evolutionarily conserved herpesviral protein interaction networks. *PLoS Pathog* **5**(9): e1000570.

Furuichi Y, Morgan M, Muthukrishnan S, Shatkin AJ (1975) Reovirus messenger RNA contains a methylated, blocked 5'-terminal structure: m-7G(5')ppp(5')G-MpCp. *Proc Natl Acad Sci U S A* **72**(1): 362-366.

Fries T, Betz C, Sohn K, Caesar S, Schlenstedt G, Bailer SM. (2007) A novel conserved nuclear localization signal is recognized by a group of yeast importins. *J Biol Chem.* **282**(27):19292-301.

Kalderon D, Roberts BL, Richardson WD, Smith AE (1984) A short amino acid sequence able to specify nuclear location. *Cell* **39** (3 Pt 2): 499-509.

Kalland KH, Ke XS, Oyan AM (2009) Tumour virology--history, status and future challenges. *Apmis* **117**(5-6): 382-399.

Kalland KH, Szilvay AM, Brokstad KA, Saetrevik W, Haukenes G (1994) The human immunodeficiency virus type 1 Rev protein shuttles between the cytoplasm and nuclear compartments. *Mol Cell Biol* **14**(11): 7436-7444.

Kates J, Beeson J (1970) Ribonucleic acid synthesis in vaccinia virus. I. The mechanism of synthesis and release of RNA in vaccinia cores. *J Mol Biol* **50**(1): 1-18.

Meyer BE, Malim MH (1994) The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm. *Genes Dev* **8**(13): 1538-1547.

Schmidt T, Striebinger H, Haas J, Bailer SM (2010) The heterogeneous nuclear ribonucleoprotein K is important for Herpes simplex virus-1 propagation. *FEBS Lett* **584**(20): 4361-4365.

Wiethoff CM, Wodrich H, Gerace L, Nemerow GR (2005) Adenovirus protein VI mediates membrane disruption following capsid disassembly. *J Virol* **79**(4): 1992-2000.

Wodrich H, Krausslich HG (2001) Nucleocytoplasmic RNA transport in retroviral replication. *Results Probl Cell Differ* **34**: 197-217.

Wodrich H, Guan T, Cingolani G, Von Seggern D, Nemerow G et al. (2003) Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. *Embo J* **22**(23): 6245-6255.

Wodrich H, Cassany A, D'Angelo MA, Guan T, Nemerow G et al. (2006) Adenovirus core protein pVII is translocated into the nucleus by multiple import receptor pathways. *J Virol* **80**(19): 9608-9618.

Wodrich H, Henaff D, Jammart B, Segura-Morales C, Seelmeir S et al. (2010) A capsid-encoded PPxY-motif facilitates adenovirus entry. *PLoS Pathog* **6**(3): e1000808.

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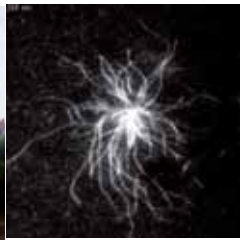

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