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Commuting within the cell—mind the GAPs

Workshop on Systems Dynamics of Intracellular Communication: Overcoming Distance in Signalling Networks

Francisca C. Bronfman[1+] *& Ruti Kapon*[2++]

[1]Pontificia Universidad Catolica de Chile, Santiago, Chile and [2]Weizmann Institute of Science, Rehovot, Israel

The EMBO/FEBS/ISF Workshop on Systems Dynamics of Intracellular Communication: Overcoming Distance in Signalling Networks took place between 18 and 22 March 2007, in the hills of Jerusalem, Israel, and was organized by M. Fainzilber, B. Kholodenko and G. Schiavo.

Keywords: intracellular communication; systems biology; transport; small G-molecules

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Introduction

The first EMBO/FEBS/ISF workshop on Systems Dynamics of Intracellular Communication: Overcoming Distance in Signalling Networks was held between the 18 and 22 of March 2007, in Maale Hachamisha, a Kibutz in the hills of Jerusalem, Israel. It brought together scientists from diverse fields of research to discuss the latest developments and theories about how information originating in the extracellular environment travels to the genome to achieve a cellular homeostatic response. Iteration between mathematical modelling and experimentation is essential to the understanding of such complex processes, but this is usually easier said than done; however, at this meeting, communication between theoreticians and experimentalists was outstanding and led to lively discussions, and to what all participants agreed was a very successful first workshop. It was decided, after a vote held at the end of the meeting, to try to organize a second meeting in 2009 at the same place; perhaps owing to the desire of some participants to follow up on the belly dancing lessons provided after the gala dinner. Here, we summarize the main topics discussed at the meeting.

Sadly, Reinhart Heinrich, who was scheduled to speak at the workshop, passed away in October 2006. In his memory, B. Kholodenko (Philadelphia, PA, USA) opened the meeting with a description of Professor Heinrich's many contributions as one of the founders of systems biology. Kholodenko followed on this theme by introducing the role of feedback loops as control mechanisms of many cellular processes. A central role for feedback loops and their modulators—most notably GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs)—in controlling intracellular communication emerged at this meeting.

Positive feedback in a control system can produce oscillations or bi-stability—the ability of an ensemble to exist stably in either of two states. Negative feedback loops produce stability and robustness or sustained oscillations when the feedback loop consists of at least three components (Kholodenko, 2006). One intuitive way of representing the response of a nonlinear system to a signal is the one-parameter bifurcation theory (Battogtokh & Tyson, 2004), which highlights qualitative changes in the behaviour of the system. J. Tyson (Blacksburg, VA, USA) described how the cell cycle could be depicted as a series of bifurcations that cause abrupt changes in the activity of Cdc2 as the cell grows (Fig 1). As the mass–nucleus ratio increases, a positive feedback loop in the cyclin kinase control network drives the cell from the G1 phase to the S/G2/M phases through saddle node/infinite period oscillation bifurcations. At the end of M phase the cell divides, which reduces the mass by half, returning

[1]Center for Cellular Regulation and Pathology (CRCP), Department of Physiology, Faculty of Biological Sciences, Pontificia Universidad Catolica de Chile, Alameda 340, Santiago, Chile
[2]Department of Biological Chemistry, Weizmann Institute of Science, 76100 Rehovot, Israel
[+]Corresponding author. Tel: +56 2 6862879; Fax +56 2 6862824;
E-mail: fbronfman@bio.puc.cl
[++]Corresponding author. Tel: +972 8 9343243; Fax: +972 8 9344128;
E-mail: ruti.kapon@weizmann.ac.il

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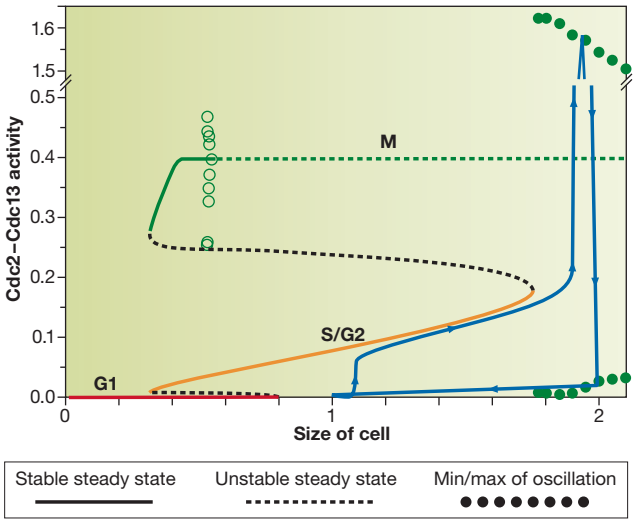

Fig 1 | Cell-cycle control represented as a bifurcation diagram. The possible states of the control system—as represented by Cdc2–Cdc13 activity—are plotted against the size of the cell—as measured by the number of ribosomes per nucleus. Steady states are indicated by solid lines and the different colours represent different stages of the cell cycle; G1 (red), S/G2 (orange) and M (green). The cell cycle starts on the left and, as the cell grows and increases in size, the control system must adjust to find the nearest stable solution. At a small cell size, three stable states separated by unstable saddle points (dashed lines) coexist. At a cell size of more than 0.8, the cell reaches a saddle node bifurcation and must leave G1 and enter S state. At approximately 1.8, another bifurcation causes it to leave G2 and enter M state; filled green circles represent maximum and minimum excursions around a stable limit cycle, and open circles around an unstable limit cycle. At the end of mitosis, the cell size is assumed to be reduced to half, placing the daughter cells at the beginning of the cycle. The superimposed blue line represents the 'cell-cycle orbit', which follows the attractors of the control system. Reprinted from Tyson *et al* (2001) with permission from the author and *Nature Reviews Molecular Cell Biology*.

the daughter cells to the beginning of the cycle. The trajectory of the cell along this diagram self-adjusts so that the average duration of the cell cycle is identical to the mass-doubling time, thereby maintaining cell size homeostasis. J. Stelling (Zurich, Switzerland) used the yeast cell cycle as a model system in which he showed that spatial sensing can be optimized by a cascade of GTPase activity with localized effector molecules. If signalling relied on the diffusion of a single GTPase then, in order to transfer information over a large distance, the GTPase gradient must be small; however, this can lead to barely interpretable spatial signals, causing a 'leak' in the network. Alternatively, a steep GTPase gradient can be generated, but this only transfers information over a short distance. Stelling proposed that a cascade of GTPase cycles, in which the GAP and GEF molecules are localized, could lead to more reliable long-range position sensing within the cell.

Designing different feedback loops for the MAPK network

Another well-known example of signalling networks is the mitogen-activated protein kinase (MAPK) pathway, which integrates external signals and propagates them from the plasma membrane to the nucleus (Kolch, 2000). As such, it regulates many fundamental cellular processes, including cell differentiation, proliferation, survival and malignant transformation. The MAPK pathway is activated by various members of the family of receptor tyrosine kinases (RTKs) such as the receptor tyrosine kinase A (TrkA) and the epidermal growth factor receptor (EGFR). Once activated, RTKs propagate the signal through a cascade of coupled phosphorylation–dephosphorylation cycles involving Ras GTPases, Raf (MAPK3), MEK (MAPK2) and ERK (MAPK). It has been known for some time that activation of TrkA by nerve growth factor (NGF) in PC12 cells—a rat adrenal pheochromocytoma cell line—triggers neuronal-like differentiation, whereas activation of the EGFR triggers cell proliferation (Marshall, 1995). This striking difference resides in the duration and intensity of ERK1/2 activity (Fig 2); however, the mechanism by which the same network achieves this differential temporal behaviour is still an enigma and was a topic of much discussion at the meeting. W. Kolch (Glasgow, Scotland) and S. Santos (Heidelberg, Germany) presented evidence suggesting that cellular outcome is determined by the dynamics of the feedback mechanisms in the network. Kolch's group used a quantitative interaction proteomics approach and found that the pathway is regulated at many levels by several feedback loops that emanate from ERK. These regulate various components of the pathway both in terms of activity as well as subcellular localization (Fig 2). The elimination of negative feedback allows EGF to cause cell differentiation. By using modular response analysis (MRA; Kholodenko *et al*, 2002) and small interfering RNA (siRNA) to systematically perturb various components of the MAPK network, Santos found that functional differences in the feedback properties of the MAPK module were brought about by negative feedback after EGF activation and by positive feedback after NGF activation. One of the positive feedback loops triggered by TrkA activation is mediated by protein kinase C (PKC) phosphorylation of Raf (Fig 2). The activation of both EGF signalling and PKC activity at the same time induces differentiation rather than proliferation. S. Kuroda (Tokyo, Japan) proposed that the differential growth-factor-dependent temporal behaviour of ERK in PC12 cells depends on the dynamics of the small GTPases Ras and Rap1, which regulate Raf activation. He used both a system-level theoretical approach and an experimental approach to show that Ras is sensitive to the rate at which the concentration of growth factors increases, whereas Rap1 activation depends on the final concentration of growth factors. Consequently, transient ERK dynamics—mediated by Ras—depend on a rapid rise in concentration of growth factors, and sustained ERK dynamics—mediated by Rap1—depend on the final concentration of growth factors (see below; Fig 2).

RTK signalling and endosomal trafficking: a close encounter

The role of E3 ubiquitin ligases and endocytosis in the regulation of EGFR signalling was discussed by A. Sorkin (Aurora, CO, USA) and Y. Yarden (Rehovot, Israel). Upon ligand binding, the EGFR is ubiquitinated and rapidly endocytosed to lysosomes for degradation. By creating EGFR-defective mutants, Sorkin showed that various degrees of ubiquitination of the receptor are responsible for endocytosis and lysosomal targeting. For example, extensive ubiquitination in lysines located in the kinase domain is needed for lysosomal targeting and degradation (Fig 2). The EGFR mutants that are not targeted for degradation accumulate in early endosomes where EGFR

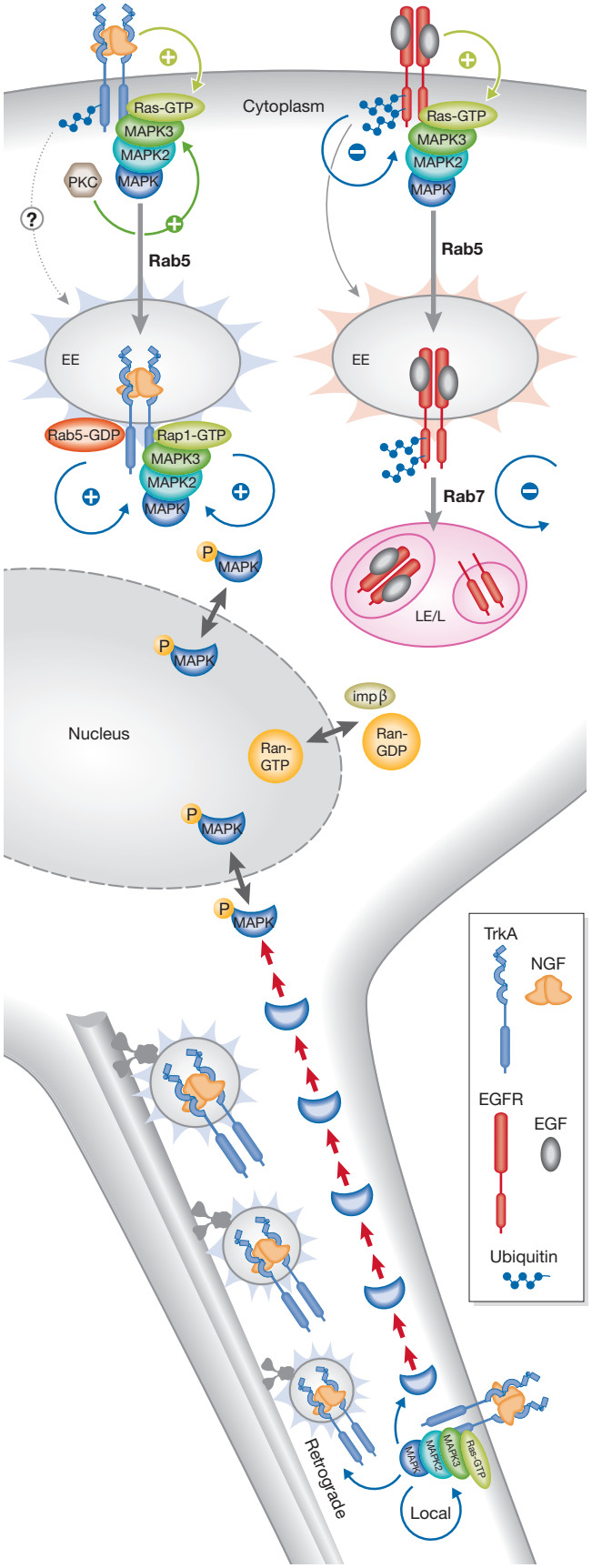

Fig 2 | Growth factors trigger local and long distance signalling. In the cell body, various growth factors, such as NGF and EGF, trigger the activation of the MAPK pathway. After the activation of TrkA (shown in blue) several positive feedback loops induce persistent MAPK signalling, causing cell differentiation. Rab GTPases regulate endosomal dynamics and signalling; for example, internalization of TrkA receptor in Rab5-positive endosomes, acts as a positive feedback loop for TrkA signalling, as shown by S. Halegoua (New York, NY, USA). Conversely, EGFR (red) is rapidly internalized to Rab7-positive endosomes in a process regulated by ubiquitination, in which EGFR signalling is rapidly downregulated. Both EGFR and TrkA are ubiquitinated and can signal from early endosomes (EE). However, how ubiquitination regulates TrkA endocytosis and why the signalling of both receptors from EE is different is still a matter of debate (see Bronfman *et al*, 2007). In distal parts of the cells, such as axons in neurons, growth factor signalling might be transmitted to the cell body by using phosphorylation waves (small red arrows) or motor-regulated vesicle transport of a signalling endosome. EGFR, epidermal growth factor receptor; imp-β, importin-β; LE/L, late endosome/lysosome; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PKC, protein kinase C; TrkA, tyrosine kinase A receptor.

is recycled quickly. Fast recycling does not necessarily result in sustained ERK activation because at steady state the EGF-binding sites in the plasma membrane remain constant. Therefore, downstream negative feedback loops might ensure downregulation of the EGFR-triggered cascade. Conversely, Yarden showed that EGFR mutants frequently found in non-small-cell lung cancer show sustained MAPK and AKT (Protein kinase B) signalling owing to impaired association of the mutant receptors with ubiquitin-protein ligase c-CBL and consequent defective endocytosis. Apparently, EGFR forms heterodimers with human epidermal growth factor receptor 2 (HER2), an oncogenic member of the EGFR family, which leads to abnormal ligand-induced ubiquitination and receptor downregulation. It is clear that, in the case of EGFR, the endocytic pathway acts as a negative feedback loop for EGF-activated MAPK. Kuroda discussed the fact that the EGFR is downregulated faster than TrkA in PC12 cells. Indeed, in the case of TrkA activation, it is already known that Rap1 activity occurs mainly in early endosomes where the concentration of NGF is constant and high (Wu *et al*, 2001). The dynamics of the endosomal network are controlled by small GTPases from the Rab family (Zerial & McBride, 2001). As discussed by M. Zerial (Dresden, Germany), the small GTPases Rab5 and Rab7 are crucial determinants of early and late endosome dynamics, organizing effector proteins into specific subdomains. Endocytic receptors reach the Rab5-positive early endosome after internalization through different mechanisms (Sorkin & Von Zastrow, 2002) whereby the early to late endosome transition seems to be controlled by a Rab5–Rab7 toggle switch that keeps the early- and late-endosomal compartments largely separate. Conversion from early to late endosome is controlled by RTK activity, which regulates downstream GAPs (Rink *et al*, 2005; Liu *et al*, 2007). Thus, RTKs might regulate the dynamics of their own endosomal transport, and the endocytic properties of each RTK will determine its final signalling outcome (Fig 2). Yarden proposed that understanding the network of negative feedback loops that regulate normal EGFR signalling might have important consequences for neoplastic therapeutic interventions.

Another new aspect of cellular signalling and intracellular vesicular transport was presented by R. Scherz-Shouval (Rehovot,

Israel). She described specific signal transduction pathways for the transformation of starvation signals into activation of the autophagy catabolic pathway in mammalian cells. She showed that reactive oxygen species, formed in the mitochondria as the result of stress, lead to the formation of autophagosomes through regulation of the activity of a cysteine protease. Thus, a localized response is formed near the mitochondria to regulate cellular homeostasis.

Nucleocytoplasmic exchange and the many roles of Ran

The ultimate consequence of a signalling cascade is to elicit a transcriptional response in the nucleus. For this to occur, the signal must cross the nuclear membrane through nuclear pore complexes.

The importance of this final leg of the route in the case of the ERK signalling cascade was presented by G.M. Ratto (Pisa, Italy). Classical ERKs come in two isoforms—ERK1 and ERK2—which are not functionally equivalent despite their high sequence homology. By using time-lapse imaging of nuclear ERK-GFP, Ratto showed that ERK2 shuttles very quickly into and out of the nucleus in its phosphorylated state (Fig 2) when compared with ERK1. As the nucleus is known to be a site of ERK inactivation by phosphatases, the ability of ERK2 to shuttle rapidly explains the sustained phosphorylation of ERK2. Constant shuttling between the nucleus and cytoplasm was a phenomenon shown by R. Kopito (Rehovot, Israel) to exist even for molecules that are considered to undergo vectorial transport. He showed that the degree of nuclear accumulation is determined by the concentration of import receptors and effectors in the cytosol. This suggests that nuclear localization signals determine the distribution of the population of molecules between the nucleus and cytoplasm, rather than the fate of the individual molecules. Along the same lines, R. Kapon (Rehovot, Israel) presented a non-equilibrium statistical mechanics model that shows how the interactions at the edges of the pore—while not strictly providing a microscopic driving force to the transport process—produce an overall accumulation when the full ensemble of transporting particles is considered. These same interactions lead to a self-regulating mechanism that can coordinate bi-directional transport through nuclear pores. 'Edge effects' are regulated through interactions with the small GTPase Ran, which regulates the affinity of cargos for their nuclear sorting receptors, such as the nuclear import factor importin-β (imp-β; Fig 2; Pemberton & Paschal, 2005). According to P. Lavia (Rome, Italy), the chromatin-centred Ran gradient model is, however, insufficient to account for all the versatility and specificity with which Ran and imp-β regulate mitotic onset and progression in mammalian somatic cells. She presented evidence that imp-β assembles into local signalling networks at specific mitotic structures, regulating interactions that are crucial to mitotic progression. M. Fainzilber (Rehovot, Israel) presented data showing that Ran regulates imp-β–dynein signalling complexes at sites in neuronal axons that are distant from cell bodies (Hanz & Fainzilber, 2006). Thus, in addition to the overall nucleocytoplasmic Ran gradient, interphase and post mitotic cells have localized mechanisms by which Ran controls critical nodes in signalling networks.

Growth factors regulating dynein retrograde transport

The question of intracellular communication is perhaps most conspicuous in neurons, which have a long process—the axon—that can extend up to several centimetres from the cell body. A long-standing question, which was addressed by several speakers, is how growth factor signalling triggered at the axonal terminal is communicated to the cell body for a transcriptional response. Kholodenko suggested that multiple positive feedback loops may give rise to travelling phosphorylation waves—with an almost constant velocity and amplitude—that facilitate rapid cell body responses to axonal-applied neurotrophins (Fig 2; MacInnis & Campenot, 2002). On the other hand, W. Mobley (Stanford, CA, USA) favoured the signalling endosome hypothesis (Howe & Mobley, 2005). Mobley presented new evidence for the movement of NGF in an endosomal vesicle along the axons of sensory neurons using biotin-NGF coupled to streptavidin quatum dots (Cui *et al,* 2007; Wu *et al*, 2007). Mobley showed that NGF moves in a retrograde manner with a stop and go movement, yet shows remarkable processivity along the axon. The data suggest that a single NGF molecule in a vesicle is sufficient to allow endosomal signalling along the axon. Along the same lines, K. Pfister (Charlottsville, VA, USA) showed that NGF increases retrograde dynein movement and that Trk signalling endosomes recruit specific dynein complexes for their transport. Together, these results suggest that neurotrophin signalling induces more processive transport of dynein-dependent cargos by increasing the amount of dynein attached to microtubules. As discussed by J. Howard (Dresden, Germany), when more dynein motors are attached to the microtubule, more force is generated, which might result in increased movement in the retrograde direction.

Rab GTPases in the control of long-range axonal signalling

The mechanism for sorting activated growth factor receptors into those that are transported retrogradely in signalling endosomes and those that are recycled and elicit local effects is largely unknown. G. Schiavo (London, UK) and his group used a labelled retrograde-transported tetanus neurotoxin fragment (TeNT-HC) to purify retrograde-transported vesicles in motor neurons. They found that Rab5 is essential for an early step in TeNT-HC sorting to retrograde transport but is absent from retrograde-moving vesicles. Vesicles positive for TeNT-HC are also positive for neurotrophin receptors. Furthermore, Rab7 was found to be necessary for the retrograde transport of TeNT-HC. By pulling down vesicles derived from brain synaptosomes using beads conjugated to the dynein adaptor Sunday Driver, V. Cavalli (St Louis, MO, USA) found that there are large Rab5–Rab11 vesicles moving in a retrograde manner, but that these are not positive for TeNT-HC. Along the same lines, S. Halegoua (New York, NY, USA) showed that, in sympathetic neurons and PC12 cells, TrkA is retrogradely transported in Rab5-positive vesicles that resemble multivesicular bodies, in a process that is dependent on a newly identified endocytic chaperone named Pincher. Together, these results suggest that there might be different retrograde carriers for various types of signalling endosome.

The local effects of target-derived growth factors were presented by R. Kuruvilla (Baltimore, MD, USA) and C. Ibañez (Stockholm, Sweden). Kuruvilla reported that Rab11-regulated recycling of TrkA is required for axonal growth of sympathetic neurons. Ibañez discussed the relationships between GDNF (glial cell line derived neurotrophic factor) receptors and various plasma membrane subcompartments defined by differential detergent solubility. He also showed how GDNF can induce synaptogenesis in hippocampal neurons or axonal pathfinding in nodose neurons through association with the GPI-anchored (GPI for glycosylphosphatidylinositol) GDNF receptor-α1 (GFR-α1) and various co-receptors. Another

local effect of a growth factor in sensory neurons was reported by J. Twiss (Wilmington, DE, USA) who showed that NGF is able to locally regulate the translation of proteins in axons—a process that first requires retrogradely transported MAPK and phosphatidylinositol-3 kinase (PI3K) signalling to the cell body. Sympathetic neurons express both p75 and TrkA NGF receptors, but while TrkA activation induces survival and axonal growth, p75 activation induces neuronal cell death. B. Carter (Vanderbilt, TN, USA) reported that the mechanism of cell death requires p75 cleavage and the generation of an intracellular COOH fragment of p75. The subcellular location of p75 cleavage seems to be the early and recycling endosomes as shown by F. Bronfman (Santiago, Chile). This raises the possibility that in sympathetic axons, p75 cleavage in the endocytic pathway generates death-signalling fragments that are transported in a retrograde manner—a possibility that remains to be studied. It is clear that there is still more to learn about how the sorting of retrogradely transported carriers occurs and how local versus retrograde signalling is regulated.

Transport-related neurodegeneration

R. Vallee (New York, NY, USA) and O. Reiner (Rehovot, Israel) introduced Lissencephaly type 1 gene (LIS1), a protein that regulates dynein, and noted that LIS1 mutations cause lissencephaly—a severe brain malformation. By using live imaging of nuclear, centrosomal and microtubule behaviours in brain slices and differentiating neurons, Vallee showed that, in addition to transport in migrating and differentiating cells, the LIS1–dynein complex has roles in nuclear, centrosome and growth cone motility. How these new functions are coordinated with retrograde axonal transport is unknown. Complementary results were presented by Reiner, who showed that MAPK2 (PAR2) regulates the interaction of LIS1 with dynein. Further studies are needed to clarify whether LIS1 also regulates transport and signalling along the axon.

E. Rugarli (Milan, Italy) suggested that the loss of axonal homeostasis causes several neurodegenerative diseases with a 'dying back' mechanism of neurodegeneration. This is the case for spastic paraplegia where a loss-of-function mutation in the gene spastin causes retrograde degeneration of the corticospinal tract. Spastin is a microtubule-severing protein, and Rugarli showed that it regulates neurite outgrowth *in vitro*. Whether spastin activity is regulated locally by growth factors and whether it is regulating vesicular trafficking in the axon remains to be determined. Another newly described process that might be upregulated during neurodegeneration is the transport of ribosomes from Schwann cells to the axon, as originally reported by Court & Alvarez (2005) and presented in this meeting by J. Van Minnen (Amsterdam, The Netherlands). Their results open new avenues as to how extracellular signalling—in this case coming from Schwann cells—might regulate axonal homeostasis.

The compartmentalization of cells necessitates the means to traffic molecules and information throughout the cell and past membrane barriers. Although the mechanisms discussed at this meeting were different in the biomolecular sense, the role of small GTPases and their regulators—GAPs and GEFs—in conferring directionality in both space and time emerged as a major determinant of the fate of signals arising both outside and inside the cell.

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