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Review

Signaling networks during development: the case of asymmetric cell division in the *Drosophila* nervous system

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ABSTRACT

Remarkable progress in genetics and molecular biology has made possible the sequencing of the genomes from numerous species. In the post-genomic era, technical developments in the fields of proteomics and bioinformatics are poised to further catapult our understanding of protein structure, function and organization into complex signaling networks. One of the greatest challenges in the field now is to unravel the functional signaling networks and their spatio-temporal regulation in living cells. Here, the need for such in vivo system-wide level approach is illustrated in relation to the mechanisms that underlie the biological process of asymmetric cell division. Genomic, post-genomic and live imaging techniques are reviewed in light of the huge impact they are having on this field for the discovering of new proteins and for the in vivo analysis of asymmetric cell division. The proteins, signals and the emerging networking of functional connections that is arising between them during this process in the *Drosophila* nervous system will be also discussed.

Branching events in developmental pathways: Waddington's contributions

The concepts of "developmental" and "genetic" pathways evolved from the prior notion of "biochemical pathway" (Wilkings, 2002). The term "biochemical pathway" was initially coined during the first third of the twentieth century in relation to the studies of specific aromatic amino acid catabolism (Beadle and Ephrussi, 1936; Ephrussi and Beadle, 1937). The simple concept of linear biochemical pathways sustained during the thirties and forties soon evolved into much more complex metabolic schemes. Indeed, during the fifties and sixties such biochemical pathways already included cycles, feedback loops and different outcomes of each individual step. The notion of biochemical pathway preceded the appearance of two new concepts in the late 1930s: the concepts of "developmental" and "genetic" pathways. Both terms were delineated by Conrad H. Waddington in his studies with the fruit fly *Drosophila melanogaster*. He defined a "developmental pathway" as the causally linked sequence of cellular and molecular events driving a developmental process. A "genetic pathway" was considered as the causal association of a sequence of gene activities that underlies the visible events in a developmental pathway (Waddington, 1940a; Waddington, 1940b; Wilkings, 2002). Waddington's studies are historically important as they constitute the first example of genetic analysis underlying a developmental process (Waddington, 1940a). But Waddington's major contribution was to introduce the concept of "branching" to the genetic pathways he described. Instead of visualizing genetic pathways as simple linear sequences, he proposed "arborized" schemes with multiple branches and bifurcations (binary choices at each step). Despite the early formulation of these schemes, for several decades little attention was paid to Waddington's novel genetic approach. Only in the seventies did the concept of the genetic pathway resurge, especially in studies using the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* (Cline, 1979; Garcia-Bellido, 1975; Hodgkin and Brenner, 1977; Lewis, 1978; Nusslein-Volhard and Wieschaus, 1980). Since then, this approach has been employed systematically in the analysis of animal development. During the past decades, many new genes have been identified and through epistatic analysis we have been able to arrange these genes in pathways. The simple and linear genetic pathways originally depicted in the early 70s have evolved into complex networks of genetic interactions with different branching points (as described by Waddington), positive and negative feedback loops and multiple nodes of crosstalk with other pathways. These complex genetic networks have been translated into complex networks of gene activities and protein–protein interactions, as is well illustrated through our current view of signal transduction pathways.

Signaling pathways in the twenty-first century: the need for an in vivo system-wide approach

Almost seventy years after Waddington's work, it has become apparent that signal transduction pathways are not simple linear cascades within the cell. Conversely, complex signaling networks with

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multiple bifurcations and nodes of crosstalk between signaling pathways are established (Barabasi and Oltvai, 2004; Jordan et al., 2000; Papin et al., 2005). While such networks must be tightly regulated to ensure specific cell responses, the mechanisms by which signal transduction pathways are integrated and modulated within such networks are still poorly understood. Theoretical modeling of networks is providing important insights into the generic principles that govern cellular networks. This mathematical approach is very useful to understand how networks function and might have formed during evolution (Barabasi and Oltvai, 2004; Eungdamrong and Iyengar, 2004; Neves and Iyengar, 2002). Nevertheless, in addition to this approach, it is fundamental to investigate how signaling networks are regulated in living cells and in specific cell contexts. It is especially important to unravel the nodes of crosstalk between signaling cascades and the feedback loops established within the networks in vivo. The emerging view of signaling pathways as part of complex signaling networks is critical for the design of new genetic screens (Friedman and Perrimon, 2007), as well as for the pharmacological manipulation of numerous pathologies including neuronal diseases and diverse types of cancer.

The development and improvement of powerful technologies during the past decades is providing the ideal framework nowadays to untangle protein networks. Indeed, genome-wide and proteome-wide approaches are highly speeding up our knowledge about a plethora of biological processes. Likewise, the implementation of new live imaging techniques is having considerable impact for the analysis of signaling networks in vivo and for unraveling the dynamic of multiple cellular events.

Genome-wide screening

Almost 200 hundreds genomes have been sequenced since 1995 (http://www.ncbi.nlm.nih.gov:80//entrez/query.fcgi?db=Genome). The implications of this knowledge are of great relevance for analyzing how signaling networks have evolved. We can now search for common components of local networks in different species and investigate the grade of conservation in the underlying molecular mechanisms that modulate those networks (Fraser et al., 2002; Jordan et al., 2003; Kelley et al., 2003; Wilkins, 2007). Elucidation of genome sequences has latterly opened a new era of biological analysis on a system-wide level.

Functional RNAi genome-wide screening

The recent creation of genome-wide RNAi screening libraries (Chang et al., 2006; Dietzl et al., 2007) is in turn facilitating enormously the development of new screening to target novel network components, both in cell culture and in vivo (Bai et al., 2008; Dworkin and Gibson, 2006; Echeverri and Perrimon, 2006; Friedman and Perrimon, 2006; Mathey-Prevot and Perrimon, 2006; Moffat and Sabatini, 2006; Norga et al., 2003; Perrimon et al., 2007). Likewise, these RNAi screening can systematically test for genetic interactions between genes functioning in key signaling pathways in development and disease (Lehner et al., 2006; Poulin et al., 2004). RNAi technology has limitations (Niwa and Slack, 2007), but knowing and using it properly can yield through information of gene function and networking.

Microarray-based analysis of gene expression

One powerful and versatile approach for a comprehensive analysis of functional networks is the transcriptome analysis or expression profiling based on microarray/DNA chip technologies (Lockhart and Winzeler, 2000). However, despite the power of expression profiling, this approach is in essence an indirect measure for biological processes (Pandey and Mann, 2000). More accurate or direct information should be obtained by the analysis of proteins and protein–protein interactions for functional prediction, especially when trying to decipher signaling networks.

Genome-wide yeast-two hybrid screening

Regardless of some disadvantages (i.e. false negatives or false positives), the yeast-two hybrid assay is one of the most efficient techniques to explore proteomes for interactions (the so-called "interactome") (Fields and Song, 1989). Specifically, the two-hybrid method has greatly contributed to the genome-wide systematic analysis of protein–protein interactions (PPIs) (Bartel et al., 1996; Ito et al., 2001; Uetz et al., 2000). The first two comprehensive PPIs maps by genome-wide two-hybrid approaches in multicellular organisms were done in *Drosophila* and in *C. elegans* (Giot et al., 2003; Li et al., 2004). These exhaustive studies provided functional predictions for a large number of unannotated proteins from these model organisms. The implementation of all these studies has been possible due to the astounding development of reliable bioinformatics tools. These tools are inherent to the yeast-two hybrid technologies to explore, visualize and analyze the large proteomic data set. Although these high-throughput screening usually fail to yield any detailed understanding of proteins' function and cellular localization, they may provide the first prediction for function and thus, an in-route to further characterization (Titz et al., 2004). The dynamic regulation of protein interactions that exists in vivo is still a challenge that the actual static predicted networks have to confront to reach a realistic understanding of biological processes. Next years may bring new high-throughput techniques as well as improved databases and visualization tools to approach the dynamic properties of PPIs on a proteome-wide scale.

Proteome-wide screening purification of protein complexes

After the yeast-two hybrid screening, different groups tried large-scale approaches to purify protein complexes from yeast using mass spectrometry (MS) (Gavin et al., 2002; Ho et al., 2002). The same attempt to isolate protein complexes on a large-scale way from multicellular organisms has not been so successful. Recent improvements in MS sensitivity and in the development of protocols may overcome some of the difficulties found in these cases. Yeast-two hybrid and mass spectrometry approaches have been shown to yield very different data set but also highly complementary (Titz et al., 2004). For example, yeast-two hybrid analyses usually detect transient interactions whereas pull-down assays followed by MS are more appropriate to detect stable interactions (Aloy and Russell, 2002). One of the high throughput experimental strategies that is particularly well suited for protein complex purification and to identify protein–protein interactions is tandem affinity purification (TAP) (Rigaut et al., 1999) followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS or liquid chromatography tandem mass spectrometry (LC-MS/MS). Both methods can be used in a complementary way to increase interactome coverage and confidence (Butland et al., 2005; Krogan et al., 2006).

Live imaging techniques

Another critical aspect to unveil functional protein networks is to analyze the expression profile of the proteins implicated in the network in vivo. The spatio-temporal colocalization of these proteins should be a prerequisite to consider PPIs functionally significative. Likewise, the dynamic evolution in vivo of their expression patterns must be taken into account. Live cell imaging, especially in vivo fluorescence imaging, has revolutionized the way in which we can now look inside the cell. The discovery and development of fluorescent proteins from marine organisms and the high

improvements in sophisticated light microscopy techniques have made possible such revolution (Stephens and Allan, 2003; Swedlow and Platani, 2002). At present, wide-field, scanning confocal, spinning disk confocal and multiphoton fluorescence microscopes constitute general tools for live cell imaging (Stephens and Allan, 2003). Some of these techniques have been successfully used to image protein–protein interactions and gene expression in living animals (Massoud et al., 2007; Ray et al., 2002). Fluorescents proteins can also be used to track the localization of proteins, organelles or any subcellular compartment and to dynamically study processes of endocytosis and intracellular protein trafficking. Certainly, live imaging has enormously impacted the way to approach the analysis and the advance of multiple biological processes, including the process of asymmetric cell division that we will next discuss.

"The case of asymmetric cell division"

Asymmetric cell division is a highly conserved mechanism to generate cellular diversity during animal development and a crucial process in stem cell biology (Chia et al., 2008; Doe, 2008; Gonczy, 2008; Knoblich, 2008; Wodarz and Huttner, 2003). A fundamental prerequisite for an asymmetric cell division is to establish an axis of cell polarity. Only in polarized progenitors can the mitotic spindle be correctly orientated and cell-fate determinants localized asymmetrically. These two events are critical and must be tightly coordinated to ensure the segregation of determinants into only one daughter cell (Fig. 1). Since the isolation of the cell-fate determinant Numb, the first protein shown to be required for asymmetric cell division about twenty years ago, an increasing number of proteins that participate in this process have been characterized (Uemura et al., 1989) (Table 1 and Fig. 2).

Invertebrate proteins and their vertebrate orthologues

Most of our knowledge about asymmetric cell division and the proteins involved in this process has come from studies in invertebrates, such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Betschinger and Knoblich, 2004). However, the sequencing of numerous genomes has facilitated the identification of mammalian homologues of those invertebrate proteins (Table 1). Moreover, many of these mammalian proteins are orthologues of their invertebrate counterparts. For example, Par proteins were first identified in *C. elegans* as essential regulators for asymmetric cell division (Kemphues et al., 1988). The discovery of these proteins was soon followed for the identification of homologues in *Drosophila* and in mammals, where the highly conserved function of this group of proteins revealed Par proteins as universal modulators of cell polarity (Goldstein and Macara, 2007; Pellettieri and Seydoux, 2002). In *Drosophila*, we can also find several examples of proteins involved in asymmetric cell division that were identified afterwards in mammals. One of these proteins is Inscuteable (Insc), whose homologue in vertebrates remained elusive for a long time. Three years ago, two groups characterized the mammalian orthologue of Insc, mInsc (Lechler and Fuchs, 2005; Zigman et al., 2005), which only shares about 20% sequence identity with Insc. Certainly, by using BLAST searches through different genomes, Zigman and colleagues identified Insc homologues in different insect species, in honeybee, pufferfish, chicken, mouse, rat and human finding low conservation between the vertebrate and the invertebrate groups. However, the region in Insc comprising the asymmetry domain, which is necessary and sufficient for all Insc functions (Knoblich et al., 1999), shows the highest homology, and genome homology search revealed this domain as a common platform for interacting partners (Zigman et al., 2005). Indeed, both works show evolutionary conservation of the interactions found in *Drosophila* between Insc and their partners Bazooka (Baz) (Par-3 in vertebrates), Partner of Inscuteable (Pins) (LGN in vertebrates) and Gα (Gα1) (Lechler and Fuchs, 2005; Zigman et al., 2005). Hence, mInsc forms analogous complexes with Par-3, LGN and Gα to those described in *Drosophila*. Homologues in vertebrates of other *Drosophila* proteins implicated in asymmetric cell division, such as the cell-fate determinant Numb, were cloned several years ago (Verdi et al., 1996; Zhong et al., 1996; Zhong et al., 1997). The vertebrate homologues of Numb, mammalian Numb and Numblike, show a conserved function during asymmetric cell division (Cayouette et al., 2001; Li et al., 2003; Petersen et al., 2002; Shen et al., 2002; Silva et al., 2002; Verdi et al., 1996; Wakamatsu et al., 1999; Zhong et al., 1996; Zhong et al., 1997). Nevertheless, controversial issues for a long time have been the localization of mammalian Numb in progenitor cells and the Numb requirement for promoting either the progenitor or the differentiation daughter cell-fate, depending of the tissue and organism analyzed (Zhong, 2003). A recent work by Rasin and collaborators may help to enlighten part of this conflicting point (Rasin et al., 2007). Using immuno-electron microscopic analysis, authors show a basolateral localization of Numb in mitotic radial glia cells (RGCs) in mice, in contrast with the apical crescents previously reported (Zhong et al., 1996). Authors observe that the

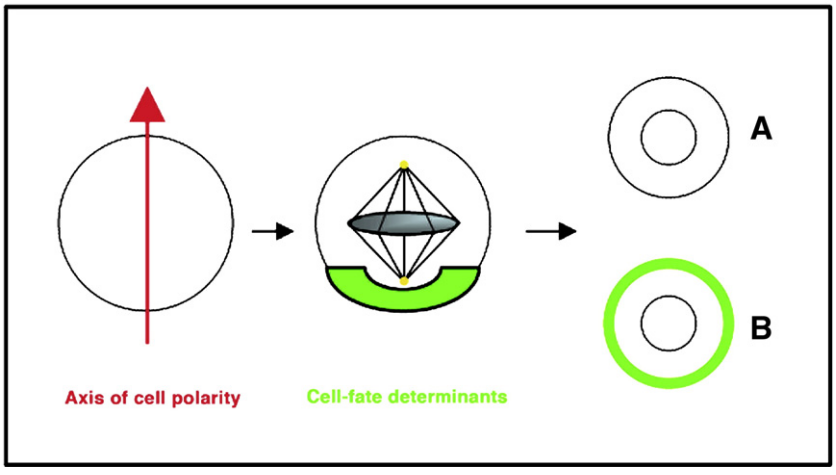

Fig. 1. Fundamental requirements for an asymmetric cell division. A prerequisite to accomplish an asymmetric cell division is to establish an axis of cell polarity. This permits the asymmetric distribution of cell-fate determinants and the proper orientation of the mitotic spindle with respect to the axis of cell polarity previously defined. These processes must be tightly coordinated to achieve an asymmetric cell division, whereby only one daughter cell receives the determinants and becomes different from its sibling.

Table 1
Proteins involved in asymmetric cell division in *Drosophila* and their homologues in *C. elegans* and mammals

Drosophila	*C. elegans*	Mammals
"Apical Proteins"		
DmPar6 (1)	PAR-6 (2)	mPAR-6 (3,4)
Bazooka (Baz) (5-7)	PAR-3 (8)	ASIP/mPAR-3 (9,10)
DaPKC (11)	PKC-3 (12)	aPKCζ (13,14)
Cdc42 (15)	CDC42 (16)	CDC42 (17)
Partner of Inscuteable (Pins) (21-23)	GPR-1/2 (24,25)	mPINS/LGN/AGS3 (10,26-30)
Gαi (22,31,32)	Gαi/o (GOA-1, GPA-16) (33)	Gαi (34)
Mushroom body defect (Mud) (35–37)	Lin-5 (25,38)	NuMA (26)
Canoe (Cno) (39)	Ce-AF-6 (40)	AF-6/Afadin (41,42)
Other G subunits and G-protein regulators		
Gαo (22,32,43)		
Gβγ (31,32)	Gβ (GPB-1) (33,44)	Gβγ (34)
Ric-8 (45-47)	RIC-8 (48,49)	mRIC-8A (50)
Locomotion defects (Loco) (51)	–	RGS12/RGS14 (52)
Myosins, kinesins, motor complexes		
Myosin VI:		
Jaguar (Jar) (53)	SPE-15 (54)	Myosin VI (55)
Myosin II:		
Zipper (Zip) (56)	NMY-2 (57)	Myh10/MYH10**
Kinesin heavy chain (Khc-73) (58)	KLP-4*	GAKIN (59)
Egalitarian (Egl)/	C10G6.1*	–
Bicaudal-D (BicD)/	C43G2.2*	BICD1**
Dynein (60)	DYNEIN (61)	DYNEIN (10)
Cell-fate determinants/adaptor proteins		
Numb (62,63)	NUM-1*	NUMB/NUMB-LIKE (64-66)
Prospero (Pros) (67-69)	CEH-26 (70)	PROX-1 (71,72)
Brain tumor (Brat) (73-75)	NCL-1/LIN-41 (76,77)	TRIM2/3/32 (78,79)
Miranda (Mira) (80-84)	–	–
Partner of Numb (Pon) (85)	–	–
Staufen (Stau) (86,87)	F55A4.5/XB646 *	h-STAU/m-STAU (88)
Tumor suppressor proteins		
Lethal giant larvae (Lgl) (89–91)	F56F10.4/XB484*	LLGL/HUGL/MLGL (92,93)
Discs Large (Dlg) (89,90,94)	DLG-1 (95)	DLG1-4 (96,97)
Scribble (Scrib) (98)	LET-413 (99)	VARTUL (100)
Cell cycle-related		
Aurora-A (Aur-A) (101-103)	AIR-1 (104)	Aurora-A (105)
Borealis (Bora)* (106)	F57C2.6 (106)	BAE24669/**
Anaphase-promoting Complex/cyclosome (APC/C) (109)	APC/C (107)	APC/C (108)
Cdc2 (110)	CDK-1*	Cdc2a/CDC2 (111)
Incenp (112)	ICP-1 (113)	INCEP (114)
Polo (115)	PLK-2*	PLK-1**
"Telophase rescue" related		
Snail (Sna) (116,117)	SNAIL-LIKE (118)	SNAIL (119,120)
Escargot (Esg)		
Worniu (Wor)		
Drosophila TNF Receptor associated protein (DTRAF1) (121)	TRF-1*	TRAF4**
Eiger (Egr) (121)	–	TNF (122)
Frizzled signaling related		
Frizzled (Fz) (123)	LIN-17, MOM-5 (124)	FZD1-10 (125)
Dishevelled (Dsh) (123)	MIG-5, DSH-2 (126,127)	DVL1-3 (128)
Strabismus (Stbm) (129)	B0410.2a (130)	VANGL1-2 (131)
Prickle (Pk) (129)	TAG-15 (130)	LMO6/PRICKLE1-2 (132)
Flamingo (Fmi) (133)	CDH-6 (130)	CELSRI-R3 (134)
Notch signaling related		
Delta (Dl)/Notch (N) (135,136)	APX-1/LAG-2/GLP-1/ LIN-12 (137)	DELTA1-2/JAGGED1-2/ NOTCH1-4 (138)
Suppressor of Hairless (Su (H)) (139)	LAG-1 (140)	CBF-1 (141)
Neuralized (Neu) (142)	F10D7.5*	NEUR1 (143)
α-Adaptin (144)	APA-2*	AP2A2**
Sanpodo (Spdo) (145,146)	TMD-1*	TMOD-1**
Sec-15 (147)	C28G1.3*	EXOC6**
Rab-11 (148)	RAB-11.1*	RAB11A**
Nuclear fallout (Nuf) (148)	F55C12.1*	RAB11-FIP4**
Others		
Tramtrack (149)	–	ZBTB8**
Cornetto (150)	–	FBF1
E-cadherin (151)	HMR-1 (152)	E-CADHERIN (153)
Abstrakt (Abs) (154)	H27M09.1*	DDX41**
Frayed (Fray) (155)	GCK-3*	OXSR1**
Mo25 (155)	MOP-25.1*	CAB39**
Lkb1 (155)	PAR-4*	STK11**

*http://www.wormbase.org/; **http://www.ensembl.org/index.html.

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apparent accumulation of Numb at the apical zone was not inside the mitotic RGCs but outside, within the apical end-feet of neighboring interphase RGCs. The closeness of these interphase RGCs gives the wrong impression of a Numb apical crescent, as previously described. Hence, given that Numb localization is also basal in *Drosophila* and chick, Numb localization would be evolutionary conserved in all these organisms (Rhyu et al., 1994; Wakamatsu et al., 1999). It is not clear though in that work whether Numb is required for the progenitor of for the differentiation daughter cell-fate (Rasin et al., 2007). The enrichment of Numb in the apical-end feet of interphase RGCs that the authors describe is related with a new function they uncover for Numb in adherens junctions maintenance and cell polarity (Rasin et al., 2007). This and other recent works have also unveiled peculiar functions for mammalian Numb and Numblike during neural development, apart to the most traditional role of Numb in cell-fate specification and Notch signaling inhibition during asymmetric cell division (Huang et al., 2005; Kuo et al., 2006; Nishimura et al., 2006; Rasin et al., 2007). Homologues of the other *Drosophila* cell-fate determinants described, the transcription factor Prospero (Pros) and the growth inhibitor Brain tumor (Brat) have been also identified. Despite that the Prospero-related homeobox 1 (Prox1) has related functions modulating the proliferation of retinal progenitors (Dyer et al., 2003), no involvement for Prox1 during asymmetric cell division has been reported, and the function of the closest mammalian homologues of Brat, the Tripartite motif proteins (Trim2, 3 and 32) is barely known (van Diepen et al., 2005). The two mammalian counterparts of Pins, LGN and AGS3, were also cloned several years ago (Mochizuki et al., 1996; Takesono et al., 1999) but their biological function was unknown until recently. A report by Sanada and Tsai shows that AGS3, a non-receptor activator of Gβγ subunits of heterotrimeric G proteins, is required to trigger Gβγ signaling in cerebral cortical progenitors. This signal is fundamental for the proper orientation of the mitotic spindle and for the asymmetric cell-fate choices of progenitors (Sanada and Tsai, 2005). LGN, which shares higher homology with Pins (46%) than AGS3, also participates as a key regulator of the mitotic spindle assembly and organization by binding the microtubule binding protein NuMA (Du et al., 2001). NuMA is among the few cases, if not the only one, of a vertebrate protein

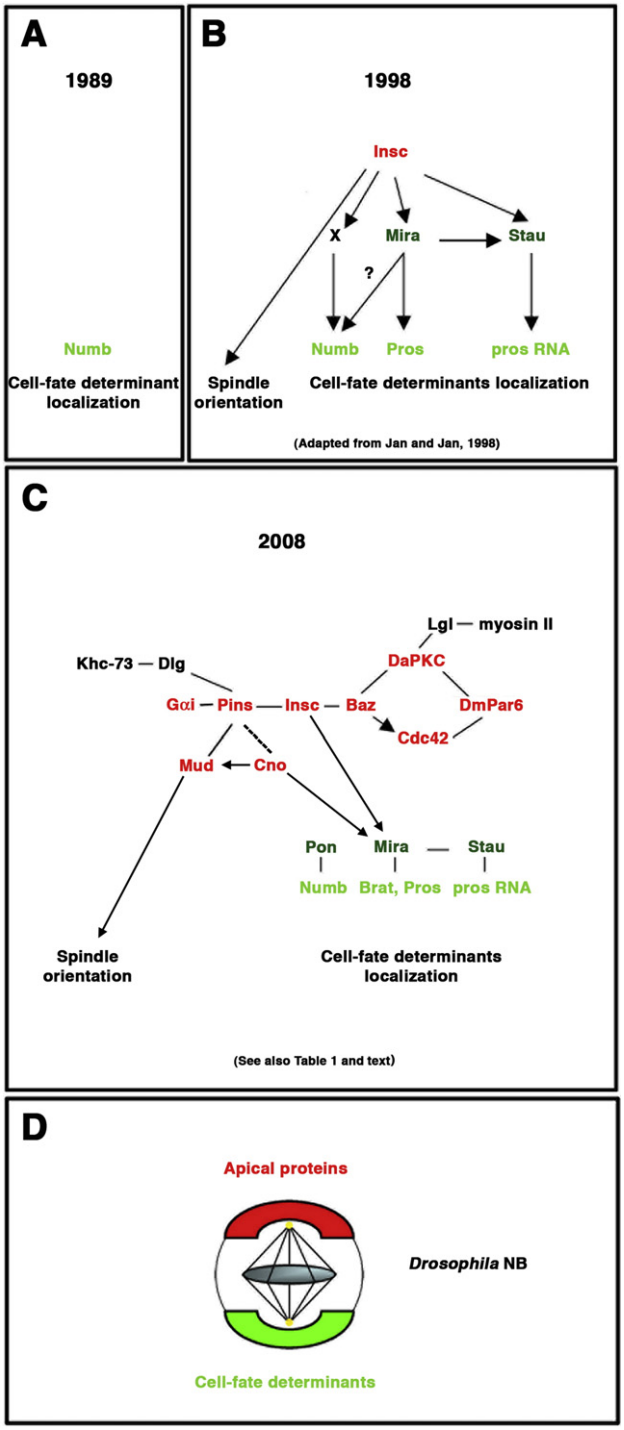

Fig. 2. The protein network associated with asymmetric cell division has increased in complexity over the past twenty years. (A) In 1989, the first protein involved in asymmetric cell division called Numb was described. (B) Nine years later, additional components formed part of a still simple network of protein interactions. (C) A very simplified view of the complex protein network that has so far been implicated in asymmetric division of *Drosophila* NBs (see also text and Table 1). (D) In metaphase NBs, cell-fate determinants and their adaptor proteins are asymmetrically concentrated at the basal pole (shown in light and dark green, respectively, in panels A–C) whereas "apical proteins" are restricted to the apical cortex (shown in red in panels B, C).

involved in asymmetric cell division whose counterpart in *Drosophila* was identified a posteriori. Different system-wide approaches, as next discussed, helped to find in this case the homologue of NuMA in *Drosophila*, a protein called Mushroom body defect (Mud) that turned to be the orthologue of NuMA (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006).

System-wide approaches during asymmetric cell division

Technical improvements in the post-genomic era have also impinged on the field of asymmetric cell division. During the past years, genome and proteome-wide approaches are being commonly employed to identify new components and to fit them into the complex network of regulatory interactions that underlie the process of asymmetric cell division. This is the case, for example, for two of the most recently identified players in this process in *Drosophila*, the growth inhibitor Brat (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006c) and the microtubule binding protein Mud (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006) (see also above). Brat was isolated by Betschinger and colleagues using TAP-tagged Miranda (Mira) constructs followed by LC-MS/MS. Mira is an adaptor protein, which binds the cell-fate determinant Pros (Table 1 and Fig. 2). Proper software (MASCOT, Matrix Science) helped to identify Brat as a new Mira-binding protein (Betschinger et al., 2006). The function of Mud in asymmetric cell division was reported simultaneously by three different groups (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). One of these groups took advantage of developed bioinformatics tools to search for NuMA orthologues in invertebrates (Bowman et al., 2006). NuMA had been described in vertebrates as a microtubule binding protein that connects the mitotic spindle with the vertebrate counterparts of the *Drosophila* proteins Pins and Gαi (Du and Macara, 2004). Hence, using different software programmes for sequence alignment, they searched *Drosophila* and *C. elegans* proteomes and identified Mud and LIN-5 as NuMA-like proteins (Bowman et al., 2006) (Table 1). Another group identified Mud using a co-immunoprecipitation approach with Pins-tagged proteins, followed by MS (Izumi et al., 2006). Finally, Siller and colleagues decided to analyze Mud in detail in the context of asymmetric cell division as Mud had been identified as a possible Pins interactor in a genome-wide yeast two-hybrid screen (Giot et al., 2003; Siller et al., 2006). Genome-wide approaches are being extremely useful also to give us deeper insights into the functional protein networks that control asymmetric cell division. In this line, a work by Choksi and collaborators identified, by a genome-wide expression profiling, the in vivo targets of the cell-fate determinant Pros through the whole *Drosophila* genome (Choksi et al., 2006). Pros is a landmark of differentiating daughter cells. Through this approach, authors show how Pros can both inhibit proliferative genes and activate genes for terminal differentiation in the daughter cell in which it is segregated (Choksi et al., 2006). Also, by means of proteomic and functional screens, a work by Wells and colleagues has identified in a systematic approach a new network of protein interactions key for the apical polarity of MDCK epithelial cells. This network, organized around the Cdc42 RhoGAP called Rich1, includes the scaffolding protein Amot, aPKC and the polarity and PDZ proteins Par-3, Pals1 and Patj, several of them central players in asymmetric cell division (Wells et al., 2006). In *C. elegans*, genome-wide RNAi screens have also been successfully approached to identify new components involved in asymmetric cell division (Kamath et al., 2003; Labbe et al., 2006).

The use of live cell imaging has definitely revolutionized the field of asymmetric cell division. The development of GFP-tagged molecules such as the cell-fate determinant Numb and its adaptor protein Partner of Numb (Pon), microtubule associated proteins, as Tau, and many others has made possible to monitor the dynamic behavior of all these proteins and the mitotic spindle throughout the whole cell-cycle in *Drosophila* (Albertson and Doe, 2003; Barros et al., 2003; Bellaiche et al., 2004; Bellaiche et al., 2001a; Berdnik and Knoblich, 2002; Hutterer et al., 2006; Kaltschmidt et al., 2000; Langevin et al., 2005; Lee et al., 2006a; Lu et al., 1999; Mayer et al., 2005; Ohshiro et al., 2000; Peng et al., 2000; Roegiers et al., 2001; Rolls et al., 2003; Siegrist

and Doe, 2005; Siller et al., 2006). This dynamic analysis of asymmetric cell division is contributing to change or to review some past hypothesis. For example, early models postulated the importance of the actin cytoskeleton to transport cell-fate determinants along the cortex. However, photobleaching experiments have shown that there is a very quick exchange of determinants between the cortex and the cytoplasm without detecting any lateral transport of segregating determinants. This study suggests that it is the differential affinity between determinants and the apical and basal cortex which is critical for their specific localization (Mayer et al., 2005). The analysis of asymmetric cell division in other organisms, including *C. elegans*, zebrafish, chick and human neural progenitor cells with in vivo time-lapse imaging is also providing important insights into the field. For example, in *C. elegans* ultraviolet laser microbeam, followed by time-lapse imaging using a wide-field microscope and deconvolution, has been used to remove the central spindle or the centrosomes in living embryos. The aim was to unveil the pulling forces that act on each spindle pole and the mechanisms that generate astral forces (Grill et al., 2001; Grill et al., 2003). These studies basically conclude that there is a stronger net pulling force on the posterior pole that is responsible for the posterior displacement of the spindle and that Gα subunit signaling pathway is contributing to create that force imbalance by generating astral forces at the posterior pole (Grill et al., 2001; Grill et al., 2003). Analysis of cell division in living zebrafish embryonic retinas using two-photon microscopy and 3D reconstructions has also revealed new key aspects of this process. (Das et al., 2003). The study shows that, contrary to past models for vertebrate asymmetric cell division, in the zebrafish retina there is no apico-basal divisions during the generation of postmitotic neurons. Instead, the orientation of cell division changes from central-peripheral to circumferential temporally correlated to a transition between symmetric and asymmetric cell division. The 3D reconstructions also show how a basal process connects mitotic cells with the basal surface opposite to the apical position where cells are dividing, and how this basal process is occasionally asymmetrically inherited by one daughter cell (Das et al., 2003). In a recent work, Wilcock and collaborators develop a novel long-term time-lapse imaging assay to visualize and follow through the cell cycle single mitotic cells in the early chick spinal cord (Wilcock et al., 2007). In this assay, after electroporating DNA into the neural tube, embryo slices were mounted and imaged on a Deltavision wide-field microscope. This novel assay revealed unexpected behaviors during neurogenic divisions, showing that the orientation of the mitotic spindle does not correlate with neuron-generating progenitors in the early phases of neurogenesis. Only at late stages of neurogenesis, terminal divisions that generate two neurons display a particular orientation of the spindle, parallel to the apical surface (Wilcock et al., 2007). Taking advantage of long-term time-lapse multiphoton microscopy, Haydar and colleagues already showed that the mitotic orientation of apical progenitors, from embryonic mouse cortex, is mostly planar throughout neurogenesis (Haydar et al., 2003). Similar conclusions reach another group working with living slices of developing mice forebrains (Konno et al., 2008). In this study, time-lapse imaging shows that progenitors with a mitotic spindle orientated parallel to the apical surface give rise to both progenitors and postmitotic neurons. Indeed, authors find that vertical divisions (spindle perpendicular to the apical surface) are very scarce. Hence, most of both neurogenic and proliferative divisions are planar. In other words, the orientation of the spindle is dispensable for the decision to proliferate or differentiate (Konno et al., 2008). Laser-scanning confocal microscopy and time-lapse video microscopy were also used by Fuja and colleagues to show the different patterns of expression of LGN and AGS3, two homologues of Pins (see also above) in human neural progenitor cells (Fuja et al., 2004). They found that LGN is asymmetrically distributed in these progenitors and segregates only to one daughter cell. AGS3 does not show this asymmetric distribution. Their studies conclude that human neural progenitor cells undergo asymmetric cell division and that the mechanisms that regulate this process in *Drosophila* and in human cells may be very similar (Fuja et al., 2004).

Live cell imaging has specially changed the view we had about the role of centrosomes during the process of asymmetric cell division (Rebollo et al., 2007; Rusan and Peifer, 2007; Yamashita and Fuller, 2008; Yamashita et al., 2007). Time-lapse recording of *Drosophila* larval neuroblasts carrying specific fluorescent reporters for microtubules, pericentriolar material and centrioles allowed Rebollo and colleagues to monitor the dynamic centrosome cycle, defining unequal functions for the two centrosomes. Indeed, they found that the two centrosomes become unequal at the beginning of the cycle: one centrosome nucleates an aster and remains at the apical position whereas the other, after losing periocentriolar material starts randomly and extensively moving within the cell. Finally, just before division, this centrosome localizes at the basal pole, recruits periocentriolar material and organizes an aster. Authors suggest that the orientation of the spindle is settled early in the cell cycle by the position of the apical centrosome. Hence, not only the polarized distribution of proteins but also the different behavior of different organelles, such as the centrosomes, contributes to the asymmetry of the *Drosophila* neuroblasts (Rebollo et al., 2007). Out of the nervous system, but in a striking parallelism, in the *Drosophila* germline stem cells the differential behavior of the two centrosomes also underlie the specific orientation of the spindle (Yamashita et al., 2007). Finally, another time-lapse analysis using 4D or 5D spinning disk confocal microscopy in *Drosophila* neuroblasts describes a novel centrosome cycle, further supporting the importance of differential centrosome behavior during asymmetric cell division (Rusan and Peifer, 2007).

At present and taking advantage of all these time-lapse and system-wide techniques, the next crucial step to fully understand the process of asymmetric cell division is to unravel the functional networks of protein–protein interactions (PPIs) established during this process, the key regulatory nodes within the networks and the possible feedback loops that fine-tune the whole process.

Protein–protein interactions (PPIs) during asymmetric cell division

Although many proteins are required for asymmetric cell division (Table 1), we do not know yet in detail how all these proteins are coordinated to control this process. Some pieces of the puzzle are probably still being ignored and these might shed light on the missing connections that underlie the process of asymmetric cell division. Nevertheless, some important interactions have been already defined between the proteins so far identified, both in the peripheral nervous system (PNS) and in the central nervous system (CNS) of *Drosophila*.

Emerging protein networks to regulate cell-fate determinants localization

PNS

"Anterior signaling complex", "posterior signaling complex" and Frizzled signaling pathway. PNS precursors divide within the plane of the epidermis with the mitotic spindle orientated along the anterior–posterior (A-P) axis. The Frizzled (Fz)/Dishevelled (Dsh) planar polarity pathway is critically involved in the asymmetric division of sensory organ precursors (SOPs) (Bellaiche et al., 2001a; Gho and Schweisguth, 1998). Fz receptor and its most proximal effector Dsh locate at the posterior pole of the precursor whereas other components of the pathway, such as the four-pass transmembrane protein Strabismus (Stbm) and Prickled (Pk), a LIM and PET-domain protein, locate at the anterior pole (Bellaiche et al., 2004) (Fig. 3A). All of them contribute to regulate the establishment of the A-P axis of cell polarity and the anterior localization of Pins and the PDZ protein Discs

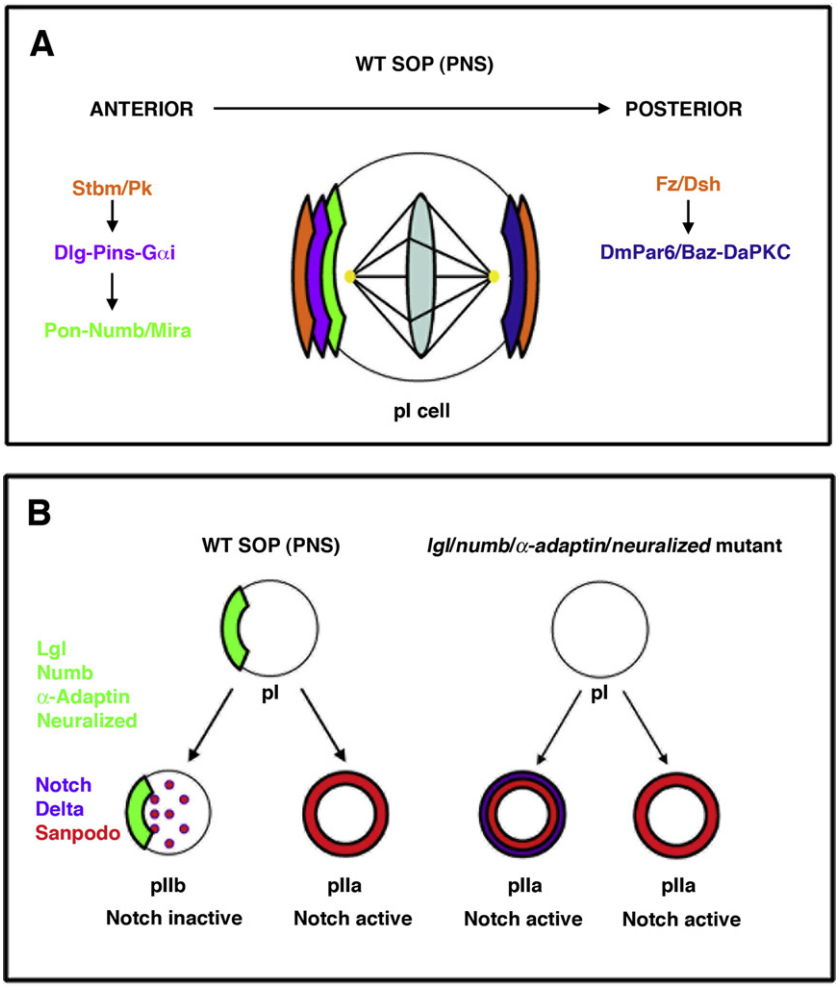

Fig. 3. Signaling networks of interactions described during asymmetric division of SOPs in the *Drosophila* PNS. (A) Fz signaling pathway (orange) regulates the localization of anterior (Dlg–Pins–Gαi) and posterior (DmPar6/Baz-DaPKC) signaling complexes. Cross-interactions between all these pathways allow the anterior localization of cell-fate determinants and adaptor proteins (Pon-Numb/Mira) and the orientation of the spindle along the A-P axis (see also text). (B) Notch signaling inactivation in the pIIb cell and activation in the pIIa cell in the PNS depends on the concerted action of multiple proteins, such as Lgl, Numb, a-Adaptin, Neuralized and Sanpodo.

Large (Dlg). Stbm binds Dlg facilitating its anterior localization (Bellaiche et al., 2004; Lee et al., 2003). Dlg, in turn, binds Pins through its SH3 domain (Bellaiche et al., 2004; Bellaiche et al., 2001b). Both proteins Dlg and Pins form part of the anterior signaling complex Pins–Gαi–Dlg required to control the anterior localization of cell-fate determinants, such as Numb and its adaptor protein Pon (Bellaiche et al., 2001a) (Fig. 3A). Fz also acts through a second signaling complex formed by Baz/DmPar6/aPKC located at the posterior cortex of the precursor (Henrique and Schweisguth, 2003) (Fig. 3A). For example, Fz cooperates with Pins to localize Baz posteriorly and Baz, in turn, regulates along with Pins–Dlg the asymmetric localization of Numb. Dlg also is required to anchor Baz at the porsterior cortex (Bellaiche et al., 2001b). Flamingo (Fmi), a seven-transmembrane cell-adhesion molecule acts downstream of Fz and is the only member of the Fz pathway whose localization in SOPs is not polarized (Lu et al., 2000). Fmi mostly locates to the cell–cell boundaries between the precursor and the surrounding cells. The localization of Numb and its adaptor protein Pon are also altered in *fmi* mutants (Lu et al., 2000).

Cell-cycle regulators

Different proteins critically involved in cell-cycle regulation have also been shown to modulate asymmetric cell division in the PNS. Such is the case for the kinases Aurora-A (Aur-A) and Cdc2 (Berdnik and Knoblich, 2002; Chia et al., 2008; Hutterer et al., 2006). Aurora kinases were first identified in *Drosophila* though they are highly conserved in all eukaryotes (Glover et al., 1995). Whereas Cdc2 kinases have a general master regulator function in cell division, the function of Aur-A kinases is more restricted to specific processes during cell-cycle, such as centrosome maturation and spindle assembly (Crane et al., 2004). During asymmetric cell division, Aur-A has an independent function regulating the localization of the cell-fate determinant Numb in the SOPs of *Drosophila* PNS (Berdnik and Knoblich, 2002). A new Aur-A binding protein called Borealis (Bora) has been described for its role in the process of asymmetric cell division (Hutterer et al., 2006). Bora is required to activate Aur-A in the cytoplasm at the onset of mitosis. However, Bora is a nuclear protein. The model proposed by Hutterer and colleagues to explain the mechanism by which these proteins are acting includes the Cdc2 kinase. Authors show how the activation of Cdc2 at the beginning of mitosis promotes the exclusion of Bora from the nucleus to the cytoplasm. Here, Bora can activate Aur-A contributing to asymmetrically locate Numb (Hutterer et al., 2006). These results link all these proteins explaining also previous data which showed that Cdc2 is essential for Aur-A activation (Marumoto et al., 2002; Maton et al., 2003).

Cell-fate determinants and Notch signaling pathway

The first relationship connecting proteins involved in asymmetric cell division was reported in 1996 between the “intrinsic” factor Numb

and the "extrinsic" factor Notch (Guo et al., 1996; Spana and Doe, 1996). These studies showed that Numb antagonizes Notch signaling through a direct protein–protein interaction in the daughter cell in which Numb is asymmetrically segregated, both in the PNS and in the CNS (Guo et al., 1996; Spana and Doe, 1996). More recent data from studies in the *Drosophila* PNS has further clarified the relationship between Numb and Notch, introducing other components that participate in and mediate this inhibitory interaction. This is the case for the endocytic protein α-Adaptin, another Notch-interacting partner, the E3 ubiquitin ligase Neuralized and the four-pass transmembrane protein Sanpodo, a positive regulator of Notch signaling (Berdnik et al., 2002; Hutterer and Knoblich, 2005). Numb, α-Adaptin and Neuralized localize to the anterior part of the SOP pI and asymmetrically segregate to the pIIb daughter cell (Fig. 3B). The actual model sustains that Numb binds α-Adaptin and inhibits Notch in the pIIb cell through the α-Adaptin-mediated endocytosis of Sanpodo, a protein required for Notch signaling (Berdnik et al., 2002; Hutterer and Knoblich, 2005). Neuralized ubiquitynates Delta in the pIIb cell leading to Delta endocytosis (Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2001). The endocytosis of the ligand Delta promoted by Neuralized is required for cleavage of the receptor Notch in the sibling cell pIIa. Consequently, Neuralized activates Notch non-autonomously in the pIIa cell (Fig. 3B) (Le Borgne and Schweisguth, 2003). In addition, more recent studies show that both Neuralized and the tumor suppressor protein Lethal Giant Larvae (Lgl) contribute to regulate Sanpodo localization in the pIIb cell (Langevin et al., 2005; Roegiers et al., 2005). Lgl also promotes the cortical recruitment of Numb and its adaptor protein Pon, further contributing to the inhibition of Notch signaling in the anterior pIIb cell (Fig. 3B) (Langevin et al., 2005).

Additional regulatory crosstalk has been described in the PNS between intrinsic factors such as the conserved protein kinase aPKC and the cell-fate determinant Numb (Smith et al., 2007). aPKC phosphorylates *Drosophila* and mammalian Numb both in vivo (HEK293T cells) and in vitro kinase assays on two serine residues, Ser7 and Ser295 (Smith et al., 2007). These sites match the consensus aPKC phosphorylation sites and are conserved throughout species. In a normal situation, Numb accumulates in the basolateral membrane of mammalian epithelial cells, opposite to aPKC, which is present in the apical domain. In *Drosophila* PNS, Numb accumulates to the anterior part of the SOPs, just opposite to the posterior localized aPKC. Mutant forms of Numb lacking those Ser residues are uniformly distributed around the cell cortex of mammalian epithelial cells and *Drosophil*a SOPs. Hence, Numb phosphorylation by aPKC constitutes a general mechanism to regulate the correct asymmetric distribution of Numb at the cortical membrane both in mammals and in *Drosophila* (Smith et al., 2007). aPKC also phosphorilates the tumor suppressor protein Lgl on three conserved serines (Betschinger et al., 2003) (see also below), restricting Lgl function to the anterior part of the SOP (Langevin et al., 2005). Here, Lgl will also contribute to Numb localization and thus to Notch inhibition, as discussed before.

CNS

Apical proteins, tumor suppressor proteins and non-muscle myosins

Regulatory crosstalk has also been described in the CNS between intrinsic factors such as the apical protein aPKC and the tumor suppressor protein Lgl. aPKC phosphorylates Lgl at the apical pole of metaphase neuroblasts (NBs) of the CNS, where aPKC is asymmetrically distributed (Betschinger et al., 2003). The phosphorylation of Lgl by aPKC induces a conformational change in Lgl that ultimately leads to its inactivation (Betschinger et al., 2005). Hence, Lgl is only active at the basal cortex where aPKC is not found. This is critical for the correct asymmetric localization of determinants to the basal pole of the NBs both in vertebrates and invertebrates (Betschinger et al., 2003; Chalmers et al., 2005; Langevin et al., 2005; Plant et al., 2003; Yamanaka et al., 2003) (Fig. 4A).

Functional relationships have also been reported between the tumor suppressor protein Lgl and non-muscle myosins, such as myosin II and myosin VI (called in *Drosophila* Zipper and Jaguar, respectively; Table 1). These myosins are required to transport cell-fate determinants and their adaptor proteins to the basal pole of the NB by a process dependent on the actin cytoskeleton (Barros et al., 2003; Petritsch et al., 2003). Lgl binds to myosin II in different organisms and this association inhibits filament formation and myosin activity (Kagami et al., 1998; Strand et al., 1994; Strand et al., 1995). As Lgl is phosphorylated and inhibited by aPKC at the apical cortex of metaphase NBs (Betschinger et al., 2003), myosin II is active in this apical domain. In contrast, Lgl binds to and inhibits myosin II at the basal cortex where Lgl is active due to the lack of aPKC (Fig. 4A). The apically restricted myosin II modifies the actin cytoskeleton at the apical cortex, avoiding the binding of determinants and their adaptor proteins there and thus, restricting them to the basal cortex (Barros et al., 2003). While this mechanism does not imply active transport, the unconventional myosin VI/Jaguar has been shown to bind the adaptor protein Miranda (Mira) and to transport it to the basal pole of metaphase NB (Petritsch et al., 2003). However, the mechanism by which myosin VI promotes this transport is not yet fully clear (Mayer et al., 2005). Other myosins might also be involved along with myosin VI in this process, acting cooperatively or competitively. Indeed, both myosin VI and myosin II co-immunoprecipitate with Mira in vivo. Given that only Mira is present in the embryonic extracts pulled down by myosin VI (myosin II is not detectable in those extracts), it has been suggested that myosin II and myosin VI might compete for Miranda binding (Petritsch et al., 2003). These studies link the requirement of Lgl with the appropriate localization of cell-fate determinants through the action of non-muscle myosins. Homologues of Lgl in vertebrates are probably also implicated in similar processes (Musch et al., 2002).

Apical proteins and cell-cycle regulators. The cell-cycle regulator Cdc2 also functions in the embryonic CNS maintaining the correct localization of the apical protein Inscuteable (Insc), though Cdc2 is not necessary for Insc initial establishment (Tio et al., 2001). Only high thresholds of Cdc2 activity allow a normal asymmetric cell division through a tight temporal regulation of Insc localization. The mechanism by which the kinase Cdc2 acts involves probably phosphorylation. However, given that the potential phosphorylation sites for Cdc2 present in Insc are dispensable for Ins localization, Cdc2 must be acting indirectly on Insc (Tio et al., 2001).

The cell-cycle regulator Aur-A not only regulates the localization of Numb in the PNS (as discussed above) but also in the *Drosophila* larval CNS (Lee et al., 2006a; Wang et al., 2006). In this system, Aur-A is forming part of a new network that includes the apical protein aPKC, Numb and Notch. Aur-A prevents aPKC localization at the basal pole. As aPKC negatively regulates Numb localization, the inhibition of aPKC by Aur-A allows the accumulation of Numb in the basal pole. Consequently, Notch signaling can be inhibited by Numb in the basal daughter cell. In *aur-A* mutants, there is an increase in larval NBs due to the deregulation of Numb localization. Indeed, Notch is hiperactivated in *aur-A* mutants. Hence, Aur-A behaves as a new tumor-suppressor gene inhibiting self-renewal and promoting cell-differentiation (Lee et al., 2006a; Wang et al., 2006).

Very recent data reveals that Numb localization may also be regulated by the phosphorylation of its adaptor protein Pon by the Polo kinase (Wang et al., 2007). *polo* is another cell cycle regulator that behaves as a tumor suppressor gene, inhibiting NB proliferation and promoting neuronal differentiation. Pon is not phosphorylated in *polo* mutants and, as a result, its partner Numb is not segregated to the basal cortex of metaphase NBs. Like Numb, the apical protein aPKC is also uniformly distributed in the cortex of *polo* mutant NBs. The consequence is that both Numb and aPKC are symmetrically distributed to the progeny of these mutant NBs (Fig. 4B). In normal circumstances, Numb inhibits Notch signaling in the daughter cell to

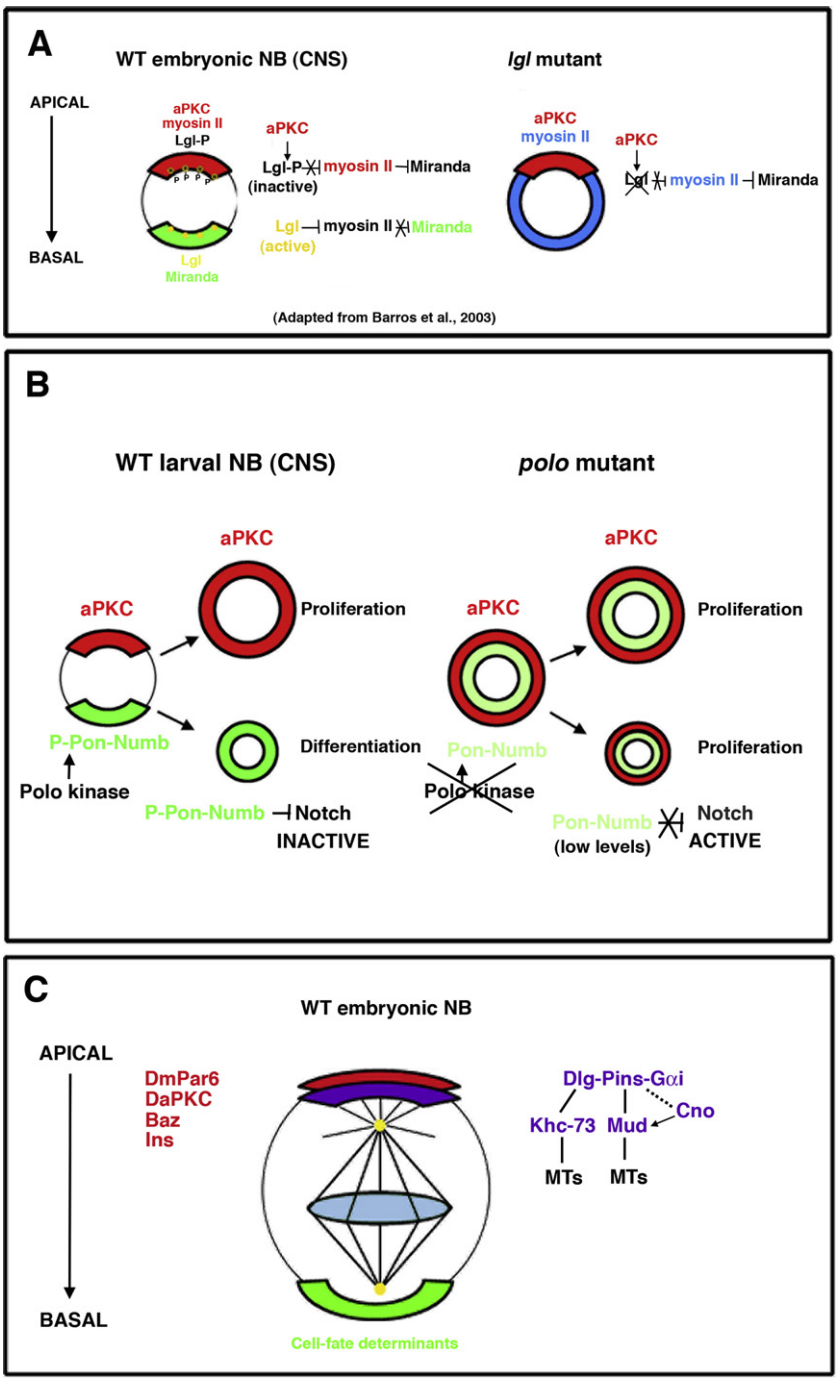

Fig. 4. Protein networks of interactions described in the *Drosophila* CNS during NBs asymmetric cell division. (A) The tumor suppressor protein Lgl interacts with the apical protein DaPKC and with myosin II to regulate the basal distribution of the adaptor protein Mira. In wt conditions, Lgl is inactivated by DaPKC apically and cannot inhibit myosin II at this location. Active myosin II excludes Mira to the basal pole where Lgl is active and can repress myosin II. In *lgl* mutants, myosin II is also active basally and inhibits Mira accumulation in this location. (B) The cell cycle regulator *polo* interacts with the apical protein DaPKC and the adaptor protein Pon to modulate the localization of the cell-fate determinant Numb. In wt larval NBs, Polo phosphorylates Pon allowing the correct distribution of its partner Numb and the inhibition of Notch in the daughter cell in which Numb is segregated. In *polo* mutants, both Pon and Numb are evenly distributed at the NB cortex. Consequently, lower levels of Numb (light green), insufficient to repress Notch, are segregated to the progeny. DaPKC localization is also affected in *polo* mutants. (C) A network of apical proteins (Cno–Pins–Gαi), tumor suppressor proteins (Dlg) and microtubule (MT)-interacting proteins (Khc-73 and Mud) coordinates the proper alignment of the mitotic spindle in CNS NBs (see also text).

which it is segregated, promoting differentiation in this cell. In *polo* mutants Numb is evenly distributed between both daughter cells and the amount of Numb segregated might be insufficient to inhibit Notch signaling, which induces cell proliferation. Additionally, aPKC is sufficient to promote cell proliferation. In wild-type conditions, only the NB daughter cell that receives aPKC maintains the capacity for self-renewal while its sibling, to which aPKC does not segregate, starts to differentiate (Lee et al., 2006b) (Fig. 4B). Hence, the symmetric distribution of aPKC to both daughter cells in conjunction with insufficient amount of Numb to inhibit Notch in either daughter cell can explain the extensive proliferation in *polo* mutations (Wang et al., 2007).

Another recent work further supports the tight correlation between the localization of components involved in asymmetric cell division and cell-cycle progression (Slack et al., 2007). The mitotic regulator anaphase-promoting complex/cyclosome (APC/C) also

functions during asymmetric cell division to basally locate the adaptor protein Mira and its cargo proteins (the cell-fate determinants Pros and Brat, the pros mRNA and the mRNA-binding protein Staufen) in the mitotic NB. The APC/C is composed by 11 protein subunits and functions as an E3 ubiquitin ligase, targeting proteins for degradation (Vodermaier, 2004). Mutations in different APC/C core subunits lead to a reduction of basal Mira and to an increase in its cytosolic accumulation. The C-terminal part of Mira is susceptible of being ubiquitylated both in cells and in larval NBs and it is necessary for Mira basal localization. However, Mira ubiquitylation has not been shown to be dependent on APC/C (Slack et al., 2007). Nevertheless, given the similar mutant phenotypes observed in APC/C mutants and in C-terminal truncated forms of Mira, it has been proposed that the reduction in Mira basal localization observed in APC/C mutants may be due to a loss of Mira ubiquitylation (Slack et al., 2007). Some Mira still remains at the basal pole in APC/C mutants, which indicates that additional or redundant mechanisms are involved in basal Mira localization. Indeed, apical proteins (such as DmPar6, Baz and aPKC) whose localization is normal in APC/C mutants (Slack et al., 2007), are also required for correct Mira localization.

Emerging protein networks to regulate mitotic spindle orientation

The asymmetric localization of cell-fate determinants in polarized progenitors must be tightly coordinated with the orientation of the mitotic spindle. Some crosstalk has also been reported between proteins involved in the proper orientation of the spindle.

PNS

The Fz signaling pathway is responsible to polarize the SOPs along the A-P axis and, consequently, to orientate the mitotic spindle along this axis (Bellaiche et al., 2001a; Bellaiche et al., 2001b; Gho and Schweisguth, 1998). The asymmetric distribution of different components of Fz pathway, such as Dsh, Stbm and Pk is key for the establishment of planar polarization. As discussed above (see also Fig. 3A), Fz signal promotes the differential distribution of Dlg–Pins–Gαi and Baz–DmPar6–aPKC at the anterior and posterior cortex of the SOP, respectively. Cross-interactions between Fz signaling components and all these proteins, as previously described, is key for the anterior–posterior orientation of the mitotic spindle (Fig. 3A; see also above).

CNS

In the CNS, the protein Pins, which binds the G i subunit of heterotrimeric G proteins through three G protein regulatory (GPR)/GoLoco motifs (Schaefer et al., 2001), forms a complex in vivo with Dlg (Siegrist and Doe, 2005). In turn, Dlg binds to the Kinesin heavy chain 73 (Khc-73), a microtubule binding protein. In this way, Dlg connects microtubules to the cortical Pins–Gαi complex that must be polarized over one spindle pole in the dividing cell (Siegrist and Doe, 2005). All these proteins are conserved in vertebrates (Table 1) so it is very likely that the interactions that occur between them are also conserved. Indeed, mammalian orthologues of Dlg have been shown to interact with microtubule-binding proteins (Brenman et al., 1998; Hanada et al., 2000; Matsumine et al., 1996; Mok et al., 2002; Passafaro et al., 1999). In addition to the Pins–Dlg–Khc-73 interactions, both Pins in *Drosophila* and its vertebrate counterpart LGN (Table 1) directly bind to another microtubule-binding protein called Mud in *Drosophila* and NuMA in vertebrates (Bowman et al., 2006; Du et al., 2001; Izumi et al., 2006; Siller et al., 2006) (Table 1). This interaction provides an additional link between microtubules and cortical proteins, ensuring the tight alignment of the mitotic spindle with the polarized cortex (Fig. 4C) (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006).

Hence, multiple intertwined mechanisms are required to ensure the correct localization of cell-fate determinants and the proper orientation of the mitotic spindle during the process of asymmetric cell division. Within each of these "local networks" of regulators different links and interconnections have been described, as previously discussed. More complex networks of protein–protein interactions may exist encompassing functional cross-interactions between different of the local networks unraveled so far. These potential connections might help to further clarify and to give new insights into the real mechanistic network underlying the process of asymmetric cell division.

PDZ domain-containing proteins: potential nodes within signaling networks?

PDZ (*P*SD-95, *D*lg, *Z*O-1) domains are among the most abundant module domains in proteins (http://www.ebi.ac.uk/interpro/DisplayIproEntry?ac=IPR001478). PDZ domains are globular structures of about 90 amino acid residues that have been traditionally involved in protein–protein interactions. These domains recognize specific short peptide sequences, normally at the C-terminus of their interacting partners (Ponting et al., 1997; Sheng and Sala, 2001). However, PDZ domains can also interact with internal protein motifs, other PDZ domains and even with lipids (Mortier et al., 2005; Nourry et al., 2003; Sheng and Sala, 2001; Wu et al., 2007; Yan et al., 2005; Zimmermann et al., 2002). Most PDZ proteins are cytoplasmic and closely associated to the plasmatic membrane at specific submembrane locations, such as cellular junctions or synapses (Ponting et al., 1997; Sheng and Sala, 2001). Multiprotein complexes can form around PDZ-based scaffolds. Through the formation of those supramolecular complexes, PDZ proteins can contribute to anchor cytosolic proteins to the plasmatic membrane, to cluster receptors and channels at the membrane and also can be decisive to increase the rate and fidelity of signal transduction. PDZ domains are often found in combination with other protein interaction domains involved in signaling events such as SH3, LIM or GUK. In addition, some PDZ proteins contain catalytic domains, which endow PDZ proteins with intrinsic enzymatic activities (Fan and Zhang, 2002). This feature further suggests that PDZ proteins can display direct and more dynamic functions regulating signaling events in addition to their more established role as static scaffolds. With all, PDZ proteins are good candidates to modulate signaling networks, acting as central nodes within particular networks (Carmena et al., 2006; Nourry et al., 2003; Sheng and Sala, 2001).

PDZ domains have been traditionally classified into three discrete functional categories depending on the C-terminus sequence of their interacting partners (Jelen et al., 2003; Sheng and Sala, 2001; Songyang et al., 1997). More classifications have been proposed as new information was obtained about the nature and structure of PDZ domains (Bezprozvanny and Maximov, 2001; Song et al., 2006). One important concern that came up with these classifications in discrete categories was the grade of promiscuity of PDZ interactions, i.e. are PDZ domains within a same class interacting with one another's ligands? A recent work in which PDZ domain selectivity is characterized and modeled on a genome-wide scale in mouse reveals that this is not the case (Stiffler et al., 2007). The model designed give accurate information about the binding selectivity of PDZ domains and the work concludes that selectivity do exist and that the intrinsic selectivity of PDZ domains is tuned across the mouse proteome to minimize cross-reactivity (Stiffler et al., 2007). Altogether, PDZ proteins may constitute specific integrative nodes within functional signaling networks.

PDZ domain-containing proteins during asymmetric cell division

It is interesting to note that several of the proteins critically involved in asymmetric cell division are PDZ domain-containing proteins, such as the conserved PAR proteins Par-6 and Baz/Par-3 and the tumor suppressor proteins Dlg and Scrib, (Table 1). All of these PDZ proteins contain additional module domains involved in protein–protein interactions (Fig. 5).

Par proteins

In *Drosophila*, two Par proteins, DmPar6 and Baz/Par-3 are key players in the process of asymmetric cell division. Both proteins localize either apically in the NBs of the CNS or at the posterior pole of the SOPs of the PNS, contributing to asymmetrically locate cell-fate determinants and to orientate properly the mitotic spindle (Fig. 2C and Fig. 3A). Par6 proteins contain three conserved regions: an amino-terminal PB1 (Phox and Bem 1) region, a CRIB (Cdc-42, Rac-Interactive Binding) motif and a PDZ domain. Baz/Par-3 protein also provides different protein–protein interfaces including three PDZ domains (Fig. 5). Baz interacts in vitro but not in vivo with DmPar6 (Petronczki and Knoblich, 2001) and binds the apical protein aPKC through a region comprising the second and third PDZ domains (amino acids 401 to 737) (Wodarz et al., 2000). In addition, Baz recruits the adaptor protein Insc to the apical pole of metaphase NB by a direct physical interaction (Schober et al., 1999; Wodarz et al., 1999). In this way, Baz links Insc and its associated partners (Fig. 2) to the apical complex DmPar6/Baz/aPKC. How does apical complex form and maintain apically? A signal emanating from the overlying neuroectoderm could initiate the recruitment of apical proteins, but so far this hypothetical signal is unknown (Siegrist and Doe, 2006). The anchorage and accumulation of apical proteins at the apical pole of the NB could be due to specific interactions with transmembrane proteins at this apical position. Another possibility is that a particular lipid fraction of the apical membrane is involved in the interaction with the apical proteins (Wodarz and Huttner, 2003). As mentioned above, PDZ proteins have also been implicated in interactions with lipids (Nourry et al., 2003). A direct binding between Baz and the lipid phosphatase PTEN, functionally related to the phosphoinositide signaling, has been reported (von Stein et al., 2005). It is not clear though the role of this interaction during the process of asymmetric cell division. A very recent work has shown how the second PDZ domain of Par3 binds to phosphatidylinositol lipid membranes, providing at least one mechanism by which the apical proteins can attach to the membrane (Wu et al., 2007). Hence, the PDZ protein Baz/Par-3 could be an important link between other apical proteins, such as Insc–Pins–Gαi, and the unknown signal that anchors all of them at the apical cortex during NB division.

The tumor-suppressor proteins Dlg and Scrib

The PDZ proteins Dlg and Scrib are also critically involved in the process of asymmetric cell division regulating basal determinants target, spindle orientation and unequal cell-sized progeny generation (Albertson and Doe, 2003; Ohshiro et al., 2000; Peng et al., 2000). Both PDZ proteins are rich in protein–protein interaction domains (Fig. 5). Dlg belongs to the Membrane-Associated GUanylate Kinase (MAGUK) homologue protein family and contains two L27 domains, three PDZ domains, one SH3 domain and a GUK domain. (http://www.ebi.ac.uk/interpro/DisplayIproEntry?ac=IPR004172) (Woods and Bryant, 1991). As discussed above, Dlg acts as a key link within different networks of proteins both in the PNS and in the CNS. In the PNS, Dlg, through its first and second PDZ domains, binds Stbm and, through its SH3 domain binds the C-terminal half of Pins (Bellaiche et al., 2001b; Lee et al., 2003). Hence, Dlg connects Stbm and Pins at the anterior pole of SOPs. In the CNS, Dlg acts as a key link between the mitotic spindle and the cortical polarity by interacting, through its GUK domain, with the microtubule binding protein Khc-73 and with Pins/Gα at the apical pole of metaphase NBs (see also above) (Siegrist and Doe, 2005). In

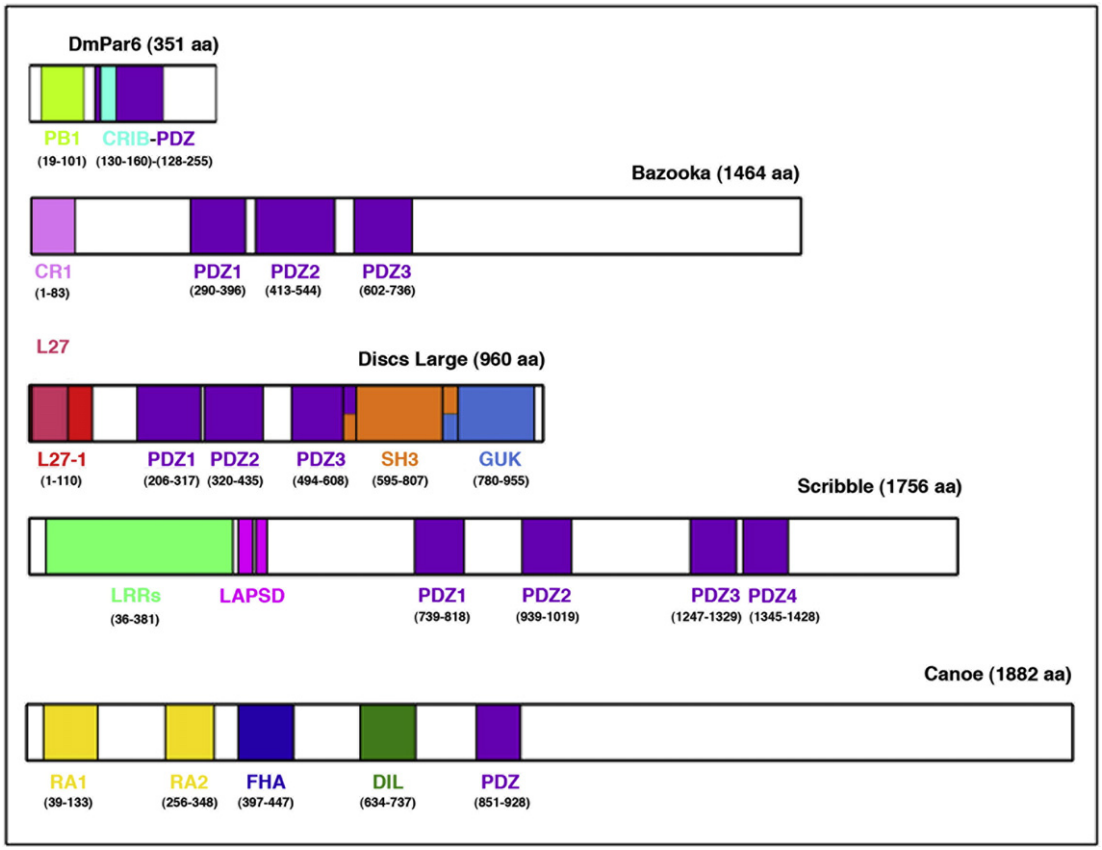

Fig. 5. PDZ domain-containing proteins involved in asymmetric cell division. The modular structure and particular domains of each protein is shown. PB1 (*P*hox and *B*em *1*); CRIB (*C*dc42/*R*ac *I*nteractive *B*inding); PDZ (*P*SD-95, *D*iscs Large, *Z*O-1); CR1 (Conserved *R*egion 1); L27 (*L*in*2*, Lin*7*); SH3 (*S*rc *H*omology region *3*); GUK (*GU*anylate *K*inase); LRRs (*L*eucine *R*ich *R*epeats); LAPSD (*LAP*-Specific *D*omain); RA (*R*as-*A*ssociation domain); FHA (*For*He*A*d); DIL (*DIL*ute). The amino acids that each domain comprises are specified inside brackets. The domains and its position have been determined according to http://www.ebi.ac.uk/interpro/, http://smart.embl-heidelberg.de/smart/, (Albertson et al., 2004; Benton and St Johnston, 2003; Peterson et al., 2004).

this work, authors show how Pins co-immunoprecipitates with Dlg from embryo lysates though they do not detect by in vitro experiments a direct physical interaction between Dlg and Pins.

The PDZ protein Scrib belongs to the LAP family, which contain leucine-rich repeats (LRRs) and multi-PDZ domains. Specifically, Scrib is a LAP4 protein that contain 16 LRRs and 4 PDZ domains (Bilder et al., 2000; Santoni et al., 2002). In addition, as all LAP proteins, Scrib has a LAP-specific domain (LAPSD), which comprises a 38 amino acids LAPSDa and a 24 amino acids LAPSDb (Santoni et al., 2002). In the CNS, Scrib has been shown to function in the same pathway with the tumor suppressor proteins Dlg and Lgl to regulate cell size and mitotic spindle asymmetry of *Drosophila* NBs (Albertson and Doe, 2003). The interaction between Scrib and Dlg is indirect, probably through the protein called Gukholder (Gukh), which, as Scrib and Dlg, is also apically enriched in metaphase NBs (Albertson and Doe, 2003; Mathew et al., 2002). Gukh binds both the GUK domain of Dlg and the second PDZ domain (PDZ2) of Scrib (Mathew et al., 2002). Despite the important role of Scrib during asymmetric cell division and cell polarity in general, not much is known about the Scrib partners and the network of functional interactions in which Scrib is involved during the process of asymmetric cell division (Albertson and Doe, 2003; Bilder and Perrimon, 2000). Some years ago, a work by Albertson and collaborators highlighted the relevance of different Scrib domains to establish cortical polarity (Albertson et al., 2004). For example, the LRR domain of Scrib is necessary and sufficient for cortical localization of Scrib in mitotic NBs and sufficient to target the adaptor protein Mira to the cortex. Likewise, PDZ2 domain is required for efficiently targeting Scrib to the apical NB cortex by binding Gukh/Dlg, as mentioned before. This PDZ2 domain, however, is not sufficient to target Scrib; the LRR domain is also required. (Albertson et al., 2004). In mammals, new Scrib interacting partners have been described over the past years. For example, the high-risk human papilloma-virus E6 protein, the guanine nucleotide exchange factor (GEF) β-PIX and the tight junction protein ZO-2 (Audebert et al., 2004; Metais et al., 2005; Nakagawa and Huibregtse, 2000). A more recent work has identified two other Scrib binding partners in mammals more closely related, in principle, to the process of asymmetric cell division, the polarity proteins Lgl2 and Vangl2 (Kallay et al., 2006). Lgl2 is the only one that binds the LRR domain of Scrib. Vangl2, localizes at the basolateral membrane of mammalian epithelial cells and binds the PDZ domains of Scrib through a C-terminal PDZ consensus-binding motif (Kallay et al., 2006).

Very recently, we have found that the *Drosophila* PDZ protein Canoe (Cno) (Fig. 5) (Miyamoto et al., 1995) functions during asymmetric cell division. Cno forms a complex in vivo with Pins and interacs genetically with other apical proteins to regulate fundamental features of an asymmetric cell division (Speicher et al., 2008). Hence, PDZ proteins such as DmPar6, Baz, Dlg, Scrib and Cno may represent good candidates as crosstalk nodes within the signaling networks established during the process of asymmetric cell division.

Feedback loops

Self-sustaining feedback loops constitute an intrinsic property of signaling networks (Bhalla and Iyengar, 1999). Feedback loops can buffer or enhance changes that occur in a system, where negative and positive feedback loops coexist in many cases. Indeed, coupled negative and positive feedback circuits have been described (Ferrell, 2002) In these circuits, positive feedback loops are fast and responsible to activate the system from “off” to “on” state (switch-like behavior) whereas negative feedback loops are delayed and responsible to restore the system to its original “off” state, avoiding an over-response (suppressors of noise effects) (Ferrell, 2002; Kim et al., 2007). The existence of multiple feedback loops contributes to the robustness of the networks (Brandman et al., 2005; Stelling et al., 2004).

Approaching the process of asymmetric cell division as a complex network of protein–protein cross-interactions will also imply unveiling the feedback loops that, as an intrinsic property of any network, must be modulating the circuit of interactions. Though not much is known at present about the existence of regulatory feedback loops during asymmetric cell division, some cases have been already reported. For example, during the division of the *Drosophila* CNS NBs a regulatory feedback loop has been proposed between the apical proteins Insc, Pins and the subunit Gαi (Schaefer et al., 2001). At the moment the NBs delaminate, Insc locates apically and, by binding Pins, Insc positions the complex Pins–Gαi to the apical cortex of the NB. At this moment the localization of Insc is completely independent of Pins or Gαi. However, once the NB has delaminated, the localization of Insc, Pins and Gαi become interdependent. In this way, the overexpression of Gαi leads to a mislocalization of Insc in the cytoplasm. Authors propose that Gαi signals through unknown downstream factors to modulate Insc apical asymmetric localization. This feedback loop would be required to maintain the NB polarity (Schaefer et al., 2001).

Another feedback loop has been recently proposed to regulate the Par complex localization, also in *Drosophila* CNS NBs (Atwood et al., 2007). In this work, Atwood and colleagues show a role for the Rho GTPase Cdc42 in NB asymmetric cell division. Cdc42 locates at the apical cortex of NBs and is required to anchor there DmPar6, through a direct interaction with its CRIB-PDZ domain, and aPKC. In *cdc42* mutants the localization of DmPar6-aPKC fails and the overexpression of Cdc42 leads to ectopic DmPar6-aPKC localization. However, Baz/Par3 localization is not altered in *cdc42* mutants. Indeed, authors find that Cdc42 acts downstream of Baz/Par3 to regulate the localization of DmPar6 and aPKC. Intriguingly, DmPar6 is in turn necessary for a strong Cdc42 apical enrichment. Hence, these results suggest that a positive feedback is established to regulate the PAR protein complex localization and activity (Atwood et al., 2007).

Regulatory feedback loops to asymmetrically locate proteins in cell polarity processes have been also described in other organisms, such as *C. elegans.* In *C.elegans*, CDC-42 is also key for PAR protein localization and acts within different feedback loops to stabilize cortical polarities. For example, CDC-42 activates myosin contractility, which in turn is required to transport CDC-42 and other factors that regulate its activity. In addition, CDC-42 directly interacts and promotes the association of PAR-6/PKC-3 at the anterior cortex of the zygote, where they restrict the localization of the PAR-2 protein to the posterior cortex. PAR-6 and PAR-2 are in turn required for the asymmetric distribution of activated CDC-42 (Aceto et al., 2006; Munro et al., 2004). In *Dictyostelium*, feedback signaling also controls the highly polarized production and accumulation of the phospholipid PtdIns(3, 4, 5)P_3 signal, which regulates the leading-edge of chemotaxing cells (Charest and Firtel, 2006). This lipid asymmetry is also a first sign of polarity in mammalian neutrophils, where PtdInsP3 and Rho GTPases are involved in a positive feedback loop required to establish neutrophil polarity (Weiner et al., 2002). With all, positive and negative feedback loops may be a general mechanism to fine-tune the regulatory networks that underlie the process of cell polarity and asymmetric cell division in many different cell types.

Conclusions and perspectives

The implementation of system-wide and live cell imaging approaches, the improvements in mass spectrometry instrumentation, as well as the development of powerful bioinformatics software is providing high-throughput technologies capable of analyzing the protein components of intracellular signaling networks. However, the task of demonstrating the physiological relevance of any given protein–protein interaction is still critical. Likewise, the computational tools developed so far have limited applications to *in vivo* systems, especially when a biological process is time-dependent or is regulated by feedback loops. Hence, the current challenge is to analyze

the functional relevance of specific protein networks during particular processes in living cells, as discussed here for the process of asymmetric cell division. Through the use of animal models specific cell contexts and temporal issues can be taken into account, crucial aspects for testing the functional significance of protein interactions. Particularly challenging in the future will be to discover and to analyze in depth the nodes of crosstalk within signaling networks, as they constitute key platforms of regulation.

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