Phosphorylation and dephosphorylation events allow for rapid segregation of fate determinants during Drosophila neuroblast asymmetric divisions

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Drosophila neuroblasts display remarkable asymmetry throughout mitosis. The most prominent asymmetry is the size difference between daughter cells at cytokinesis. The larger cell retains stem cell identity, i.e., remains a neuroblast while the smaller cell, called a ganglion mother cell (GMC), will generate differentiated neural and glial progeny. Preceding this size difference, several protein complexes localize to opposite sides of the neuroblast cortex (apical and basal in the embryo and, by analogy, referred to as such in larval neuroblasts although their asymmetry no longer correlates with such axis). The plane of division is coordinated with this molecular asymmetry such that apical and basal complexes are unequally partitioned between the two daughter cells: apical complexes are inherited by the self-renewing neuroblast while basal complexes are inherited by the GMC. This unequal segregation has been extensively shown to be functionally significant. Apical complexes contain factors required for cellular self-renewal and basal complexes contain factors required for the differentiation of the GMC progeny. Curiously, however, some “basal” neuroblast proteins such as the scaffold protein Miranda (Mira) and its associated fate determinant Prospero (Pros), are initially apically localized prior to translocating to the opposite side of the cell cortex by the onset of mitosis. This is because mira mRNA is apically enriched, where it remains throughout the cell cycle, suggesting that Mira protein is translated within the apical environment.1,2 The transition from apical to basal enrichment of Mira and Pros takes place within minutes.2 Here, we summarize the known phosphorylation events and roles during neuroblast asymmetric divisions, as well as very recent work, including our own, identifying the first protein phosphatases implicated in this process. We then discuss models previously proposed, as well as a new model, for apical-to-basal transition of the Mira complex in light of our new results.

Introducing the Apical and Basal Protein Complexes

At late interphase/onset of mitosis two apical complexes and two basal complexes are formed in the neuroblast. One of the apical complexes comprises the evolutionarily conserved partitioning defective (Par) protein complex, consisting of Par-3 (called Bazooka, Baz, in Drosophila), Par-6 and aPKC. This complex is linked by the apically restricted protein, Inscuteable, to another protein complex containing Partner of Inscuteable (Pins), the small inhibitory G protein, Gαi and Locomotion defects (Loco). The Par complex regulates segregation of the two basal complexes. One of these consists of Mira and its cell fate determining cargo, Insucutable, to another protein complex containing Partner of Insucutable (Pins), the small inhibitory G protein, Gαi and Locomotion defects (Loco). The Par complex regulates segregation of the two basal complexes. One of these consists of Mira and its cell fate determining cargo, which includes the homeodomain transcription factor Pros as well as the pros mRNA-binding protein Staufen (Stau) and the translational repressor Brain Tumor (Brat). The other basal complex contains the Notch antagonist Numb and its scaffold binding protein, Partner of Numb (Pon).3 The
segregation of the apical and basal complexes is an F-actin-dependent process as actin depolymerizing drugs abolish this process (whereas microtubule depolymerizing drugs do not).4

Neuroblast Asymmetry is Regulated by Phosphorylation

A few protein kinases have been identified to play essential roles in regulating self-renewal of Drosophila neuroblasts. These include the apical complex member aPKC, the cyclin-dependent kinase cdc2 and two other mitotic kinases, Aurora-A (AurA) and Polo. Particular phosphorylation events influence protein-protein interactions and subcellular localizations and provide mechanistic insights into the establishment of neuroblast asymmetry.

The apical kinase aPKC and the cytoskeletal protein Lethal (2) giant larvae (Lgl) regulate each other through mutual feedback. Lgl directly inhibits the kinase activity of aPKC and, conversely, aPKC negatively regulates Lgl by directly phosphorylating it on three evolutionarily conserved Serine residues (S656, S660 and S664) resulting in a conformational change, which renders it inactive. The output of these interactions restricts aPKC localization to the apical cortex and active Lgl to the basal side of the neuroblast. Other targets of aPKC have been identified to include Numb, Pon and Mira.7-9 Much like with Lgl, the aPKC-mediated phosphorylation events lead to cortical displacement of the substrates ultimately restricting the proteins to a domain complementary to that of aPKC, i.e., at the basal cortex (see discussion in final section).

Neuroblasts mutant for aurA display disrupted localization of both aPKC and Numb, resulting in a dramatic upregulation of Notch expression and a failure of daughter cells to differentiate.6,10 Par-6 was subsequently identified to be the AurA target that mediates this phenotype. At the onset of mitosis, AurA phosphorylates Par-6 at Serine 34, relieving its inhibition of aPKC. aPKC then phosphorylates Lgl, resulting in its release from the aPKC/Par6 complex and, consequently, from the apical cortex. Upon release of phosphorylated Lgl, Baz can associate with the aPKC/Par6 complex and this changes the substrate specificity of aPKC from Lgl to Numb.8

The mitotic kinase Polo has also been reported to inhibit self-renewal of Drosophila neuroblasts through the targeted phosphorylation of Pon. Polo phosphorylation of Pon at Serine 611 is both necessary and sufficient for restricting Pon, and consequently Numb, to the basal cortex.11 The G/M mitotic kinase, cdc2, has been shown to be necessary for the maintenance of the apically localized machinery during neuroblast divisions12 but the substrates involved in this process have yet to be determined.

...And by Dephosphorylation

Given the prevalence of phosphorylation events in orchestrating neuroblast asymmetry, it seemed likely that dephosphorylation steps would be equally important in its regulation. However, only this year did the first reports emerge of protein phosphatases implicated in asymmetric division of neuroblasts. One contribution to this delay relative to kinases might have been the indirect way in which protein phosphatases attain substrate specificity. Phosphatases act as obligatory multimeric complexes, where substrate specificity is conferred on the catalytic subunit by its association with one or more, often alternative, regulatory subunits.

The first of these reports was our own work, which showed that during neuroblast asymmetric divisions the evolutionarily conserved Protein Phosphatase 4 (PP4) protein complex regulates the localization specifically of the Mira complex.13 Disrupting the PP4 regulatory subunit Falafel (Flfil) results in the displacement of all Mira complex members from the cortex to the cytoplasm during mitosis, but the localization of apical protein complexes as well as of the Pon/Numb complex appears unaffected. Similar results were obtained by attenuating the activity of another regulatory subunit or of the catalytic subunit of PP4. We further showed that, although Flfil is a nuclear protein prior to nuclear envelope breakdown (NEB), nuclear localization of Flfil is not necessary for correct Mira localization at metaphase. Moreover, membrane-tethered Flfil is sufficient to rescue the localization of the Mira complex during mitosis. We therefore favour a model in which after NEB, PP4 acts in the cytoplasm or at the membrane to perform a dephosphorylation step required for association of Mira with the basal cortex.

Our phenotypic data and binding assays suggested Mira itself as a likely target for PP4-mediated dephosphorylation but we were unable to demonstrate that Mira is indeed a direct target of PP4. We showed that Mira and Flfil co-immunoprecipitate from larval extracts. In addition we showed that Mira directly interacts with Flfil in a yeast two-hybrid assay, via a previously mapped domain that is necessary for its cortical association.14 Furthermore, in the same forward genetic screen (Sousa-Nunes R and Somers WG, unpublished), we uncovered an allele of Mira that specifically disrupts this domain and which results in a mislocalization phenotype analogous to that of flfil mutants.

A subsequent study from the laboratory of Kenneth Prehoda nicely complemented our own by revealing that aPKC is capable of both directly binding to as well as phosphorylating the same cortical association domain of Mira.9 As alluded to above, aPKC phosphorylation of Mira excludes it from the cell cortex. Our data would suggest that dephosphorylation of cytoplasmic Mira would then enable it to bind to the basal cell cortex. It remains to be shown whether PP4 can dephosphorylate Mira, in particular on the residues targeted by aPKC, but this seems a likely scenario.

Interestingly, the cortical localization of Pros has also been correlated with its phosphorylation state. Cortically associated Pros is highly phosphorylated compared to nuclear localized Pros.15 Phosphorylation is believed to control its subcellular localization as a result of conformational changes that expose otherwise hidden nuclear export sequences. We did not detect any Pros/Flfil complex formation in vivo nor in vitro and although Pros can potentially be recruited onto the PP4 complex by its association with Mira, via Flfil, this is unlikely to mediate Pros dephosphorylation given that nuclear Pros can be found in cells where Flfil is either present (early born neurons) or absent.
(fl+ neuroblasts). Potentially Pros could be a target for the PP4 complex, but if so this would more likely be mediated through an alternative regulatory subunit rather than by Fl+. At this point, neither the kinase(s) not the phosphatase(s) that modify Pros are known.

More recently still, two studies identified the Protein Phosphatase 2A (PP2A) complex as an important regulator of self-renewal of Drosophila neuroblasts. Disruption of the PP2A complex results in the mislocalization of both aPKC and Numb throughout the cell cortex. It was also observed that although PP2A-depleted tissue has lower overall levels of phosphorylated Numb, the aPKC-dependent phosphorylation of Serine 52 of Numb is specifically enhanced. This suggests that Serine 52 may be a direct target for PP2A dephosphorylation or else that it is an indirect target for a PP2A-dependent process. Similarly, lower levels of phosphorylated Numb were also observed in polon mutant neuroblasts, prompting speculation that PP2A functions to activate Polo kinase to then phosphorylate both Pon and Numb. Polo kinase has been reported to be a phosphorylation target of AurA in human cells, and it will be interesting to determine whether the actions of AurA and PP2A regulate Polo kinase to coordinate asymmetric divisions and regulate self-renewal of neuroblasts.

**Models for Apical-to-Basal Translocation of Mira and Associated Proteins**

The rapid apical-to-basal translocation of the Mira complex occurs just before the onset of mitosis. This is seen by colocalization of low-level asymmetric Mira and Insc in a very low percentage of neuroblasts that are invariably negative for the mitotic marker phospho-Histone H3 (PH3) (Sousa-Nunes R and Somers WG, unpublished observations). As soon as neuroblasts become PH3-positive, i.e., by prophase, Insc and Mira are seen to accumulate on opposite sides of the cell. In addition to cortical Mira, non-cortical Mira is most apparent during prophase and pro/metaphase; this includes cytoplasmic Mira at a higher level but also nuclear Mira. However, by metaphase most non-cortical Mira has been cleared and the Mira basal accumulation is at its most robust.

Two models have so far been proposed to explain the rapid apical-to-basal translocation of the Mira complex, each of which implicates a distinct Myosin. The time of apical-to-basal translocation of Mira is also the time that Myosin II (Myo II) becomes enriched in a cortical domain complementary to that of Mira, although Myo II and Mira localizations are initially not strictly mutually exclusive (Sousa-Nunes R and Somers WG, unpublished observations). According to one model, Mira would be displaced within the cortex, “pushed” by Myo II. However, the role of Myo II has recently been questioned following the discovery that the drug used in the previous study to inhibit Myosin II activity had a greater affinity for aPKC, which it also inhibits. According to another model, Mira would travel via the cytoplasm, in association with another Myosin, Myo VI (called Jaguar, Jar, in Drosophila). However, more recent work from the same author and collaborators, using fluorescence recovery after photobleaching (FRAP) analyses of a Mira::GFP fusion protein, suggests that the kinetics of the movement of the fusion protein in vivo is more consistent with passive diffusion than with motor-assisted transport.

What role does that leave for Jar? And does movement both via the cortex and via the cytoplasm occur? Furthermore, does nuclear/cyttoplasmic shuttling of Mira also occur in this transition and if so, would it contribute to the correct Mira localization at metaphase?

Our own data indicates that one or more dephosphorylation step(s) by PP4 must occur for Mira to stably associate with the basal cortex. Our data together with that of the Prehoda lab is suggestive of a model whereby aPKC phosphorylates newly-translated Mira, releasing it from the apical cortex; following NEB, Fl+ and or the PP4 complex is released into the cytoplasm where it can target cytoplasmic Mira; we speculate that PP4 thus dephosphorylates cytoplasmic Mira at key residues that allow it to associate with the cortex; this association would necessarily be basal given the presence of aPKC apically. The net effect of these phosphorylation and dephosphorylation events is apical-to-basal translocation of Mira, and, as a consequence, of the Mira-associated proteins. Release of Mira on one side of the cell and capture of other Mira molecules on the opposite side could lead to a very fast net translocation, faster than the translocation of each individual Mira molecule.

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