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Asymmetric localisation of Miranda and its cargo proteins during neuroblast division requires the anaphase-promoting complex/cyclosome

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Asymmetric cell divisions generate cell fate diversity during both invertebrate and vertebrate development. *Drosophila* neural progenitors or neuroblasts (NBs) each divide asymmetrically to produce a larger neuroblast and a smaller ganglion mother cell (GMC). The asymmetric localisation of neural cell fate determinants and their adapter proteins to the neuroblast cortex during mitosis facilitates their preferential segregation to the GMC upon cytokinesis. In this study we report a novel role for the anaphase-promoting complex/cyclosome (APC/C) during this process. Attenuation of APC/C activity disrupts the asymmetric localisation of the adapter protein Miranda and its associated cargo proteins Staufen, Prospero and Brat, but not other components of the asymmetric division machinery. We demonstrate that Miranda is ubiquitylated via its C-terminal domain; removal of this domain disrupts Miranda localisation and replacement of this domain with a ubiquitin moiety restores normal asymmetric Miranda localisation. Our results demonstrate that APC/C activity and ubiquitylation of Miranda are required for the asymmetric localisation of Miranda and its cargo proteins to the NB cortex.

KEY WORDS: Miranda, Anaphase promoting complex, Asymmetric division, Neuroblast, *Drosophila*

INTRODUCTION

Drosophila embryonic neuroblasts (NBs) divide asymmetrically along the apicobasal (A/B) axis to generate a larger apical NB and a smaller basal ganglion mother cell (GMC). A protein complex forms at the apical cortex of the NB and directs both the basal localisation of neural cell fate determinants and reorientation of the mitotic spindle to ensure that cell fate determinants are segregated into the GMC upon cytokinesis (Kaltschmidt et al., 2000; Jan and Jan, 2001; Wang and Chia, 2005). An apically localised protein cassette consisting of Par3 (Bazooka), Par6 and atypical protein kinase C (aPKC) recruits Inscuteable (Insc), Partner of Inscuteable (Pins, also known as Raps) and the G protein subunit Gαi to form an apical crescent at late interphase/early prophase (Kraut and Campos-Ortega, 1996; Kraut et al., 1996; Schober et al., 1999; Parmentier et al., 2000; Schaefer et al., 2000; Wodarz et al., 2000; Petronczki and Knoblich, 2001). During late prophase/early metaphase, cell fate determinants and their respective adapter proteins Prospero-Miranda, *prospero* mRNA-Staufen, Brat-Miranda and Numb-Partner of numb (Pon) localise as crescents at the basal NB cortex (Ikeshima-Kataoka et al., 1997; Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1998; Betschinger et al., 2006; Lee et al., 2006).

Basal but not apical crescent formation requires the cortically localised tumour suppressor proteins Discs large (Dlg, also known as Dlg1) and Lethal giant larvae [Lgl, also known as L(2)gl] (Peng et al., 2000; Albertson and Doe, 2003). The precise mechanism whereby basally localised proteins are transported to the basal NB cortex is not known, but requires the activity of both myosin II and VI (Barros et al., 2003; Petritsch et al., 2003). Also, the mechanism by which the basal crescent is anchored to the NB cortex, although shown to require an intact actin cytoskeleton, remains elusive (Shen et al., 1998). Although NBs divide in the larval central brain without a fixed A/B orientation, we will continue to refer to Miranda, Pon, Numb, Pros as basal proteins and Insc, Pins and the Par proteins as apical proteins.

The localisation of basal proteins is clearly tightly coordinated and linked to changes in the cell cycle, but how this coordination is achieved is not known. In this study we have identified a novel role for a key mitotic regulator, the anaphase-promoting complex/cyclosome (APC/C), in regulating the basal localisation of Miranda. The APC/C is a multi-subunit protein complex with at least 11 core subunits that functions as an E3 ubiquitin ligase (Vodermaier, 2004) normally targeting proteins for degradation via the 26S proteasome (Holloway et al., 1993). Regulation of APC/C activity by transient associations with the activating subunits Cdc20 and Cdh1 promotes mitotic transitions via several key processes, including the destruction of mitotic cyclins and inhibitors of chromosome separation as well as the regulation of DNA replication, centrosome duplication and mitotic spindle assembly (Sigrist et al., 1995; Zur and Brandeis, 2001; Leismann and Lehner, 2003). More recently, APC/C activity has been shown to play important roles in cell cycle-independent processes, including the control of axon growth and patterning in the developing mammalian brain (Konishi et al., 2004) and the regulation of synaptic size and transmission in both *Caenorhabditis elegans* and *Drosophila* (Juo and Kaplan, 2004; van Roessel et al., 2004). A role for the APC/C has been described in establishing the anteroposterior axis of the *C. elegans* embryo by asymmetrically distributing PAR proteins and promoting the association of the paternal pronucleus/centrosome with the actin-rich cortex (Rappleye et al., 2002). We describe a novel role for the APC/C in mediating NB asymmetric division in *Drosophila* by regulating the asymmetric localisation and/or retention of Miranda and its associated cargo proteins at the NB cortex.

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MATERIALS AND METHODS

Flies

ida^{PL17} was isolated from a genetic screen (Slack et al., 2006). Other stocks used in this study include *cdc27*^{makos} (D. Glover, University of Cambridge, UK), *mor*^{3} and *mor*^{5} (T. Orr-Weaver, MIT, Cambridge, MA), *lgl*^{4} (C. Doe, University of Oregon, Eugene, OR), *cdh1*^{rapie28} (C. Lehner, University of Zurich, Switzerland), *miranda*^{RR127} and *Df(3R)ora*^{19} (F. Matsuzaki, RIKEN, Kobe, Japan). *cnn*^{HK21}, *fzy*^{1}, *ida*^{D14}, *Rpn6*^{k00103}, *Tbp-1*^{04210b}, *Df(3L)Exel6098* and *polo*^{9} were all obtained from the Bloomington stock centre. NB mitotic clones were generated using the MARCM system as previously described (Lee and Luo, 2001).

Cloning and constructs

The *ida* and *miranda* coding sequences were amplified by PCR from 0 to 24 hour embryonic cDNA and subcloned into the pENTR/D/TOPO vector (Invitrogen). Expression constructs were generated using the Gateway Cloning System (Invitrogen) and vectors from the Terrence Murphy collection distributed by the *Drosophila* Genomics Resource Center (DGRC). Full details on the cloning of constructs are available on request. The pCasPer-*hsp70*-HA-ubiquitin construct was a gift from A. Sehgal (University of Pennsylvania Medical School, Philadelphia, PA).

Antibodies and immunofluorescence

For immunofluorescence, third instar larval brains were dissected in PBS and fixed in 4% formaldehyde for 20 minutes. Depolymerisation of microtubules was carried out by incubating dissected brains in 10 mM colchicine in PBS for 1 hour prior to fixing. Actin filaments were disrupted by incubation in 200 μM latrunculinB (Sigma) in PBS for 1 hour prior to fixing. Antibodies used were mouse and rabbit anti-Miranda (F. Matsuzaki), rabbit anti-Insc, rabbit anti-Pins, rat anti-Brat, mouse anti-Prospero (Developmental Studies Hybridoma Bank, DSHB), rabbit anti-aPKC (Santa Cruz), rabbit anti-Numb and rabbit anti-PON (Y. Jan, UCSF, San Fransisco, CA), rabbit anti-Staufen (Cai Yu, Temasek Lifesciences Laboratory, University of Singapore), rabbit anti-CNN (T. Kaufman, Indiana University, Bloomington, IN), rabbit anti-Nuf (W. Sullivan, UCSC, Santa Cruz, CA), mouse anti-rab11 (Calbiochem), mouse and rabbit anti-βGal (Promega and Cappel), mouse and rabbit anti-GFP (Sigma and Molecular Probes), mouse and rabbit anti-FLAG (Sigma), rabbit anti-phosphohistone H3 (Upstate Biotechnology), mouse anti-ubiquitin FK2 (Biomol), mouse anti-α-tubulin (Sigma) and mouse anti-γ-tubulin (Sigma). Secondary antibodies conjugated to either Alexa-Fluor-488 or Alexa-Fluor-546 were from Molecular Probes. DNA was visualised using ToPro-3 (Molecular Probes) and tissue was mounted in Vectashield (Vector Labs). Immunostainings were analysed using laser scanning confocal microscopy (Zeiss LSM 510).

S2 cell transfections and immunoprecipitations

Drosophila Schneider (S2) cells were cultured in *Drosophila* serum-free media (Invitrogen) and transfections were carried out using the Cellfectin transfection reagent (Invitrogen) according to the manufacturer's instructions. For heat-shock treatments, cells were incubated at 37°C for 2 hours and then at 25°C for 4 hours before lysis. Cultured cells and larval brains were lysed in IP lysis buffer (150 mM NaCl, 100 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100) containing protease inhibitor cocktail (Calbiochem) and the proteasome inhibitor MG115 (Sigma). Cell extracts were immunoprecipitated using rabbit anti-FLAG (Sigma) or rabbit anti-HA (Sigma) antibodies and immunocomplexes were bound to protein G sepharose (Roche). Bound complexes were washed with IP lysis buffer and subjected to immunoblotting using appropriate antibodies.

RESULTS AND DISCUSSION

Disruption to APC/C activity causes Miranda localisation defects

We have recently isolated a novel allele of *imaginal discs arrested* (*ida*), homozygotes of which survive until early pupal stages of development and fail to properly localise Miranda to the basal cortex of mitotic neuroblasts (Slack et al., 2006). Sequence analysis of this allele, *ida*^{PL17}, revealed a single nucleotide transversion within the *ida* coding sequence, resulting in a premature stop codon at aa 334 (Q334→stop), the first residue of a putative tetratricopeptide (TPR) motif. In mutant larvae, only 58% (n=72) of prophase NBs properly localised Miranda to the cortex (Fig. 1B) compared with 100% (n=64) of wild-type prophase NBs (Fig. 1A). A total of 41% (n=209) of *ida*^{PL17} metaphase NBs showed Miranda accumulation in a pericentrosomal compartment at the expense of cortically localised protein, with 8% (n=209) of mutant NBs showing a complete loss of cortically localised protein (Fig. 1D,N), whereas 91% (n=197) of wild-type metaphase NBs showed cortically localised protein only (Fig. 1C,N). We observed, at low frequency, anaphase cells as defined by separated chromosome populations displaced towards opposite poles of the cell, confirming previous observations (Bentley et al., 2002) that *ida* mutant neuroblasts are not arrested at metaphase. Miranda was still mislocalised to pericentrosomal regions in these anaphase neuroblasts (Fig. 1I). We did not observe any phenotypic defects in *ida*^{PL17} homozygous mutant embryos, presumably due to the perdurance of maternally provided protein, and NB clones induced at early larval stages did not show any obvious mitotic or Miranda-localisation defects, again indicative of protein stability (data not shown). Attempts to induce maternal germline clones homozygous for *ida*^{PL17} did not yield any surviving embryos, suggesting an essential requirement for *ida* function during oogenesis (data not shown). The *ida*^{PL17} mutant appears to be a genetic null, because the penetrance of the Miranda localisation phenotype did not increase in hemizygotes over a small deficiency that removes the entire *ida* locus [*Df(3L)Exel6098*; 42% of NBs with pericentrosomal Miranda, n=235] or in transheterozygotes over the mRNA null allele, *ida*^{D14} (43% of NBs with pericentrosomal Miranda, n=223) (Fig. 1N). We were able to rescue the defects in Miranda localisation in *ida* mutant NBs by expressing a GFP::Ida fusion protein, which, at all stages of the cell cycle, was localised throughout the cytoplasm (Fig. 1E and data not shown).

ida encodes the *Drosophila* homologue of human APC5, a subunit of the APC/C multiprotein complex (Bentley et al., 2002). In order to determine whether the defects in Miranda localisation are a specific consequence of *ida* loss of function or are caused by a more general disruption to APC/C activity, we analysed the effect of loss-of-function mutations in genes encoding other APC/C subunits. We observed a significant number of mitotic NBs with pericentrosomal Miranda accumulation in animals homozygous for mutations in either *cdc27* (13%, n=119; Fig. 1F) or *morula* (*APC2*; 39%, n=128; Fig. 1G), suggesting that Miranda asymmetric cortical localisation is disrupted when APC/C activity is attenuated. However, we did not see any defects in Miranda localisation in homozygous mutants for strong loss-of-function alleles for the two APC/C activators, *cdc20* or *cdh1*, indicating that Miranda targeting might occur independently of these two proteins (data not shown). Loss of *ida* function causes several mitotic defects, including an increased mitotic index, loss of cyclin B degradation and hypercondensed chromosomes (Bentley et al., 2002). However, a strong hypomorphic allele of *polo* that shows similar mitotic defects had normal cortical Miranda localisation (data not shown). Furthermore, colchicine treatment of wild-type NBs to depolymerise microtubules and induce metaphase arrest did not disrupt Miranda localisation, therefore suggesting that the *ida* mutant phenotype is not a secondary consequence of a delay or block in mitosis.

In *C. elegans*, APC/C function during embryonic anteroposterior axis formation promotes the association of the paternal pronucleus/centrosome with the embryonic cortex (Rappleye et al., 2002). The pericentrosomal accumulation of Miranda in APC/C mutant NBs led us to investigate a possible requirement for centrosomal function during Miranda localisation. We examined loss-of-function mutants

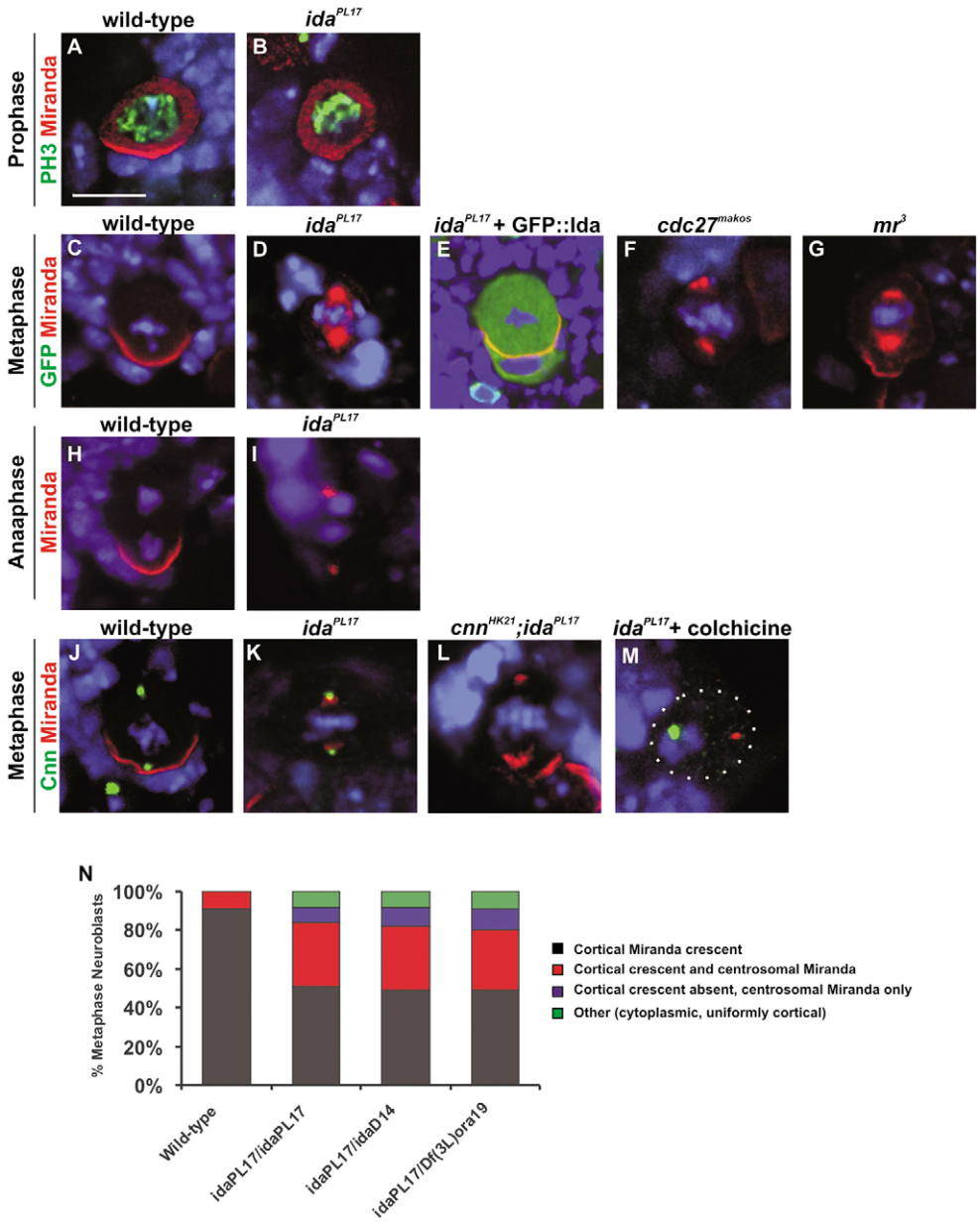

Fig. 1. APC/C function is required for Miranda localisation to the basal cortex. (**A-D**,**H**,**I**) In *ida*^{PL17} mutant neuroblasts (NBs), Miranda is exclusively cytoplasmic during prophase (B) and accumulates pericentrosomally during metaphase (D) and anaphase (I), rather than forming a cortical crescent as in wild-type larval NBs (A,C,H). (**E**) Expression of a GFP::Ida fusion protein fully rescues the Miranda localisation defects observed in *ida*^{PL17} mutant NBs. (**F**,**G**) Loss-of-function mutants for APC/C subunits *cdc27* (F) and *morula* (*mr*, *APC2*; G) also show Miranda pericentrosomal accumulation. (**J-M**) Miranda mislocalisation occurs independently of both centrosome function and microtubules. *cnn; ida* double mutants still accumulate pericentrosomal Miranda (L) and Miranda mislocalisation in *ida* mutant NBs is insensitive to colchicine treatment, which depolymerises microtubules (M). Broken circle in M indicates the outline of the NB. Miranda, red (A-M); PH3, green (A,B); GFP, green (E); Cnn, green (J-M); DNA, blue (A-M). (**N**) Quantification of Miranda pericentrosomal mislocalisation in allelic combinations of *ida* mutants. Scale bar: 10 μm.

for *centrosomin* (*cnn*), which encodes a core component of the centrosome in *Drosophila* and is required for proper centrosome assembly (Megraw et al., 1999), and found that Miranda localisation to the NB cortex was normal in these mutants (data not shown). Furthermore, *cnn; ida* double mutants still accumulated pericentrosomal Miranda, suggesting that the *ida* mutant phenotype is not dependent on intact centrosomal function (Fig. 1L). We also note that we did not see complete colocalisation of Miranda with Centrosomin in *ida* mutant NBs, suggesting that Miranda is localised in a separate compartment that itself localises to a region near to the centrosome. Accumulation of Miranda in *ida* mutants was insensitive to colchicine treatment to depolymerise microtubules, suggesting that the *ida* mutant phenotype is not dependent on the mitotic spindle, although the compartment in which Miranda localises separates from the region of the centrosome upon colchicine treatment (Fig. 1M). We also observed pericentrosomal accumulation of Miranda in *ida* mutants after latrunculin treatment, suggesting that the *ida* mutant phenotype occurs independently of intact actin filaments (data not shown).

We have so far been unable to identify this pericentrosomal compartment, although we did not observe colocalisation with either Rab11 or Nuclear fallout (Nuf), both of which are markers for recycling endosomes, suggesting that pericentrosomal Miranda accumulation occurs independently of the recycling endosomal machinery (data not shown). Miranda has been shown to localise to the centrosome both in biochemical and immunohistochemical studies (Schuldt et al., 1998; Mollinari et al., 2002). Under the conditions used for our experiments, we did not detect significant levels of centrosomal Miranda localisation in wild-type NBs.

ida/*APC5* is required for basal localisation of Miranda and its cargo proteins, but not for PON or Numb localisation or apical complex formation

The localisation of Miranda to the NB basal cortex requires the correct localisation of the apical protein complex, which includes Inscuteable, Pins and aPKC. In *ida* mutant NBs, Inscuteable localises normally to the apical cortex during early prophase and is maintained as an apical cortical crescent during metaphase (Fig.

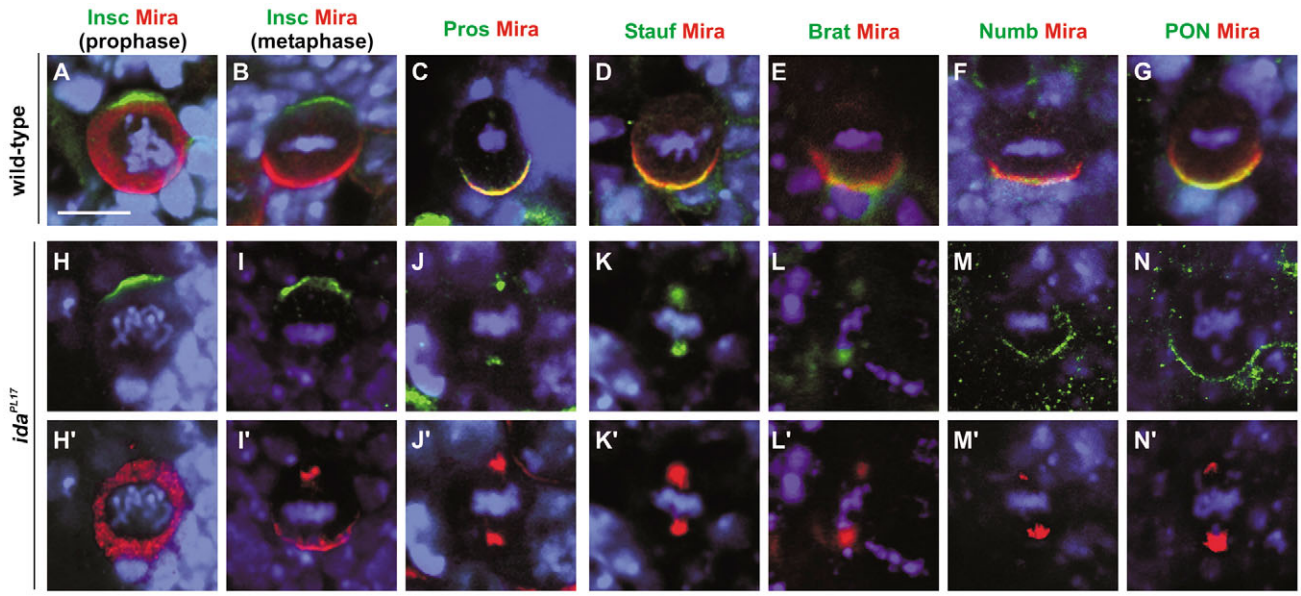

Fig. 2. Ida functions specifically to control the cortical localisation of Miranda and its associated proteins, but not to control PON/Numb. (**A-G**) Wild-type localisation of Inscuteable (Insc, green; A,B), Prospero (Pros, green; C), Staufen (Stauf, green; D), Brat (green; E), Numb (green; F), PON (green; G) and Miranda (Mira, red; A-G) in mitotic larval neuroblasts (NBs). (**H**,**I**) Inscuteable (green) localisation is unaffected in *ida* mutant NBs and a cortical Inscuteable crescent is always seen. Where Miranda (red in H′,I′) crescents form, Inscuteable and Miranda crescents occupy opposite sides of the cell cortex (I,I′). (**J-L**) Prospero (green, J), Staufen (green, K) and Brat (green, L) accumulate pericentrosomally with Miranda (J′-L′) in *ida* mutant NBs. (**M**,**N**) By contrast, Numb (green, M) and PON (green, N) remain associated with the NB cortex even in *ida* mutant NBs that show strong Miranda mislocalisation defects (M′,N′). Miranda localisation is shown in red (H′-N′); DNA in blue. Scale bar: 10 μm.

2H,I). Similar results were obtained using anti-aPKC and anti-Pins (data not shown), suggesting that the defects in Miranda localisation in *ida* mutant NBs is not caused by a disruption to the apical complex and that the APC/C functions downstream of or in parallel to the apical components.

Miranda acts as an adapter protein for the cell fate determinant Prospero, for the *prospero* mRNA adapter Staufen and for the translational repressor, Brat. In *ida* mutant NBs, Prospero, Staufen and Brat all lose their cortical localisation and colocalise with Miranda pericentrosomally, suggesting that they are still able to complex with Miranda (Fig. 2J-L). By contrast, the cortical localisation of both Numb and PON was unaffected by loss of *ida* function, and both Numb and PON formed normal cortical crescents in *ida* mutant NBs that showed strong Miranda mislocalisation defects (Fig. 2M,N), suggesting that *ida* function is required specifically for the localisation of Miranda and its associated cargo proteins to the NB basal cortex.

The *ida* mutant phenotype resembles that seen in mutants for the tumour suppressor *lgl*, in which the targeting of Miranda and other basally localised molecules to the NB cortex is disrupted so that Miranda no longer forms a cortical crescent at metaphase but is instead mislocalised to the centrosomes and mitotic spindle (Ohshiro et al., 2000; Peng et al., 2000). We reasoned that, if the *ida* mutant phenotype resulted from a reduction of Lgl function, then further reducing Lgl activity should enhance the Miranda mislocalisation phenotype. However, removing one copy of *lgl* using a null allele had no effect on the *ida* mutant phenotype and the number of NBs with pericentrosomal Miranda was comparable to mutants for *ida* alone (49% of NBs with pericentrosomal Miranda, n=103), suggesting that Lgl is not a downstream effector of Ida activity. By contrast, removing one copy of *miranda* using a deficiency that removes the entire *miranda* locus [$Df(3R)ora^{19}$] in the ida^{PL17} mutant background strongly suppressed the Miranda mislocalisation phenotype (20% of NBs with Miranda around the centrosomes, n=98), suggesting that Miranda itself might be a target for APC/C activity.

The C-terminal domain of Miranda is required for its ubiquitylation and efficient cortical localisation

Our data demonstrates that the correct localisation of Miranda to the basal NB cortex requires APC/C. Because the APC/C normally functions as an E3 ubiquitin ligase, we tested whether this role of the APC/C could be mediating the effects on Miranda localisation. In order to determine whether Miranda can be ubiquitylated, we performed immunoprecipitations on protein extracts of *Drosophila* S2 cells in which we constitutively expressed FLAG-tagged Miranda and expressed HA-tagged ubiquitin under the control of a heat-shock promoter. After immunoprecipitation using anti-HA antibody, we were able to detect FLAG-Miranda in the immune complex in extracts only from cells in which HA-ubiquitin expression had been induced by heat shock (Fig. 3A). We performed further immunoprecipitations on protein extracts both from S2 cells and larval brains expressing only FLAG-Miranda without expressing exogenous HA-tagged ubiquitin. In both cases, we were able to detect ubiquitylated Miranda in the immune complex after immunoprecipitation using anti-FLAG antibodies (Fig. 3B,C). These results using brain and S2 extracts clearly demonstrate that Miranda can be ubiquitylated both in vivo and in S2 cells. Although the antibody used recognises both mono- and poly-ubiquitin conjugates, we only observed a single band on the western blots probed for ubiquitylated Miranda. The absence of higher molecular weight Miranda species, even in the presence of proteasome inhibitors, suggests that Miranda might be mono- rather than poly-ubiquitylated.

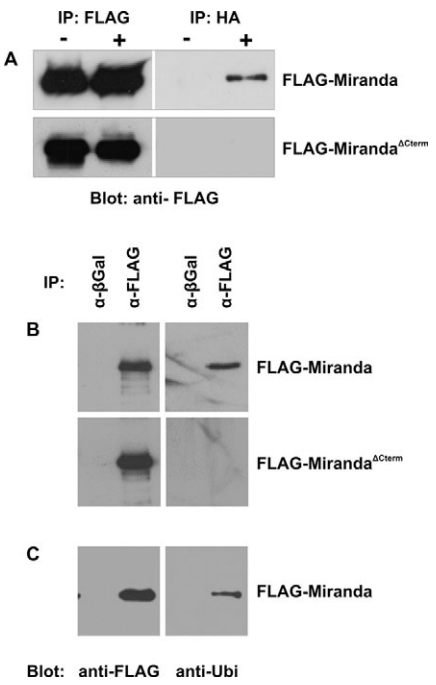

Fig. 3. The C-terminal domain of Miranda is required for its ubiquitylation. (**A**) Constructs encoding FLAG-Miranda or FLAG-Miranda in which the C-terminal 103 amino acids were deleted (FLAG-Miranda$^{\Delta Cterm}$) under the control of an actin promoter were cotransfected into *Drosophila* S2 cells with DNA encoding HA-ubiquitin under the control of a heat-shock promoter. Extracts were prepared from cells both without (–) and with (+) heat shock. FLAG-Miranda but not FLAG-Miranda$^{\Delta Cterm}$ was detected in the immune complex after the cells were heat shocked. (**B**) FLAG-Miranda and FLAG-Miranda$^{\Delta Cterm}$ were expressed in S2 cells, and cell extracts were subjected to immunoprecipitation (IP) with either anti-FLAG or anti-βgal antibodies followed by detection of ubiquitylated protein by western blotting using anti-ubiquitin antibody. Ubiquitylated Miranda was detected in the immune complex after immunoprecipitation of FLAG-Miranda but not FLAG-Miranda$^{\Delta Cterm}$. (**C**) FLAG-tagged Miranda was expressed in larval neuroblasts (NBs), and larval brain extracts were immunoprecipitated with either anti-FLAG or anti-βgal antibodies; ubiquitylated protein was detected using anti-ubiquitin antibody.

The C-terminal region of Miranda contains a putative APC/C-recognition motif (amino acids 811 to 814: GKEN) that shows homology to the KEN box, a motif required for Cdh1-dependent APC/C-mediated ubiquitylation of substrate proteins (Castro et al., 2003). To examine the effects of the removal of this motif on Miranda localisation, we used the *miranda^{RR127}* allele, in which the C-terminal 103 amino acids of the encoded protein, including the GKEN motif, are replaced by an unrelated stretch of 112 amino acids (Ikeshima-Kataoka et al., 1997). We examined the localisation of this truncated form of Miranda in mitotic larval NBs by generating somatic clones using the MARCM system, which allows the generation of homozygous-mutant NB clones that express membrane-bound CD8::GFP in an otherwise heterozygous background (Lee and Luo, 2001). In *miranda^{RR127}* mutant NBs, Miranda localisation was similar to that seen in *ida* mutant NBs: the truncated protein was exclusively cytoplasmic during early prophase (5/5 NBs; Fig. 4B,B′) and accumulated in a pericentrosomal compartment at the expense of cortical protein during metaphase (20/20 NBs; Fig. 4D,D′). As in *ida* mutant NBs, accumulation of Miranda in *miranda^{RR127}* mutant NBs was insensitive to colchicine treatment to depolymerise microtubules (Fig. 4E,E′) and we also observed accumulation of pericentrosomal Prospero in these mutant NBs (Fig. 4F,F′). Removal of this C-terminal domain prevented ubiquitylation of Miranda in S2 cells (see Fig. 3A,B). Furthermore, NBs that overexpress this truncated protein (20%, *n*=30) showed a similar mislocalisation of the expressed protein (data not shown) and replacement of this C-terminal domain with ubiquitin restored normal localisation (Fig. 4G,G′). Interestingly, mutation of the GKEN motif itself did not prevent Miranda ubiquitylation and had no effect on the localisation of the protein (data not shown), suggesting that mutation of this site alone is insufficient to disrupt ubiquitylation of Miranda.

We also observed a high proportion of embryonic *miranda^{RR127}* mutant NBs in which Miranda was localised pericentrosomally at the expense of cortical protein (90%, *n*=105; Fig. 4L). The observations that both embryonic and larval NBs mutant for the *miranda^{RR127}* allele showed some cortical localisation although at a much reduced level and the presence of low levels of cortical Miranda in embryos derived from *miranda^{RR127}* germline clones (Fig. 4M) suggest that the mutant protein produced by the *miranda^{RR127}* allele retains some ability to localise to the cortex. Ubiquitylation is clearly an important aspect of the Miranda localisation process, but in its absence a proportion of the Miranda present in the NB can be localised at the basal cortex by other mechanisms. Hence, the inability to ubiquitylate Miranda in the *miranda^{RR127}* mutant causes only an incomplete loss of localisation.

Ubiquitylation by the APC/C normally targets proteins for degradation via the 26S proteasome. Although it is possible that a proportion of ubiquitylated Miranda is targeted for degradation, disruption to proteasome function caused markedly different phenotypes than those observed when APC/C activity was attenuated. Although we observed Miranda accumulating in the region of the centrosomes in NBs mutant for the proteasome regulatory subunits *Rpn6* or *Tbp-1* (Fig. 4H,H′ and data not shown), this process was microtubule dependent, whereas in both *ida* mutant NBs and NBs mutant for the *miranda^{RR127}* allele, accumulation of Miranda was observed even after microtubule depolymerisation with colchicine (Fig. 4I,I′, compare with Fig. 1M). In addition, we did not see pericentrosomal accumulation of either Prospero or Staufen in proteasome-mutant NBs (Fig. 4J,J′ and data not shown). Furthermore, we did not observe any significant differences by western blot in Miranda protein levels between wild-type and *ida* mutant brain extracts (see Fig. S1 in the supplementary material), suggesting that the pericentrosomal localisation of Miranda in *ida* mutants is not caused by excessive Miranda accumulation and therefore reflects the disruption of a process other than proteasomal degradation. Recently, several proteasome-independent processes regulated by ubiquitylation have been identified, including protein kinase activation, vesicle trafficking, DNA repair and gene silencing (Sun and Chen, 2004).

The asymmetric localisation of cell fate determinants during NB division is tightly coordinated with changes in the cell cycle. The formation of an apical complex of proteins during early prophase not only directs the correct orientation of the mitotic spindle during metaphase but is also required for the formation of a basal crescent of cell fate determinants and their adapter molecules during late prophase/metaphase (Kaltschmidt et al., 2000; Jan and Jan, 2001; Wang and Chia, 2005). It thus appears likely that multiple components of the cell cycle machinery that coordinate cell cycle transitions might also be involved in the regulation of basal protein localisation, as has been previously shown for *cdc2* (Tio et al., 2001)

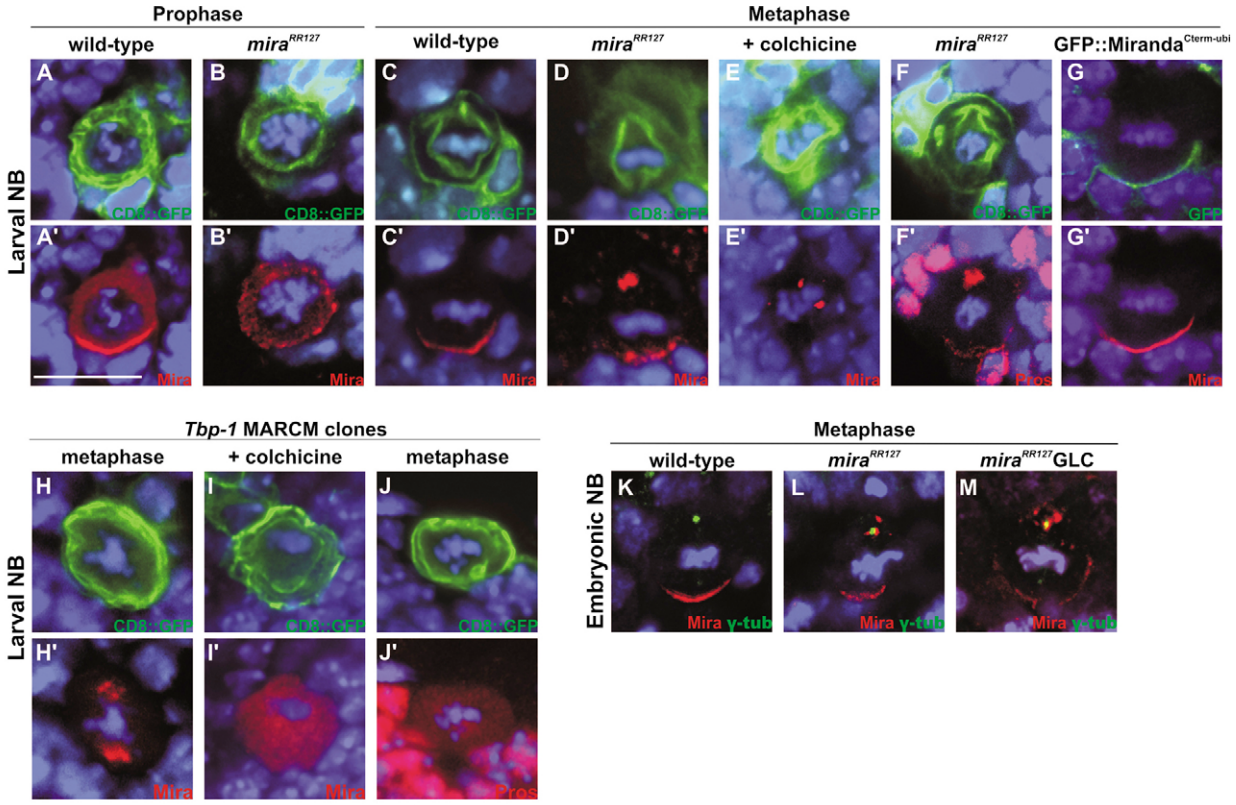

Fig. 4. The C-terminal region of Miranda is required for its correct cortical localisation. (**A-F'**) Larval neuroblast (NB) clones generated using the MARCM system. NB clones are labelled with CD8::GFP (green, A-F), Miranda (red, A'-E') and Prospero (red, F'); DNA (blue). *miranda*^{RR127} mutant NBs do not properly localise Miranda to the cell cortex during prophase (B,B') and accumulate both Miranda (D,D') and Prospero (F,F') pericentrosomally at the expense of cortical protein during metaphase. Miranda mislocalisation in *miranda*^{RR127} mutant NBs occurs independently of microtubule function and is still observed after colchicine treatment to depolymerise microtubules (E,E'). (**G**,**G'**) Replacement of the C-terminal domain of Miranda in *miranda*^{RR127} mutants with ubiquitin restores normal protein localisation. GFP::Mira$^{\Delta Cterm\text{-}ubi}$, green; Miranda, red. (**H**,**H'**) NB clones mutant for the proteasome regulatory subunit *Tbp-1* show pericentrosomal accumulation of Miranda. (**I**,**I'**) However, after treatment with colchicine to depolymerise microtubules, Miranda disperses throughout the cytoplasm (compare to E,E'). (**J**,**J'**) Prospero is uniformly cytoplasmic in *Tbp-1* mutant NBs (compare to Fig. 2I). (**K-M**) Miranda localisation in *miranda*^{RR127} stage 9 NBs. Miranda is mislocalised pericentrosomally in a high proportion of *miranda*^{RR127} zygotic (90%, *n*=105; L) and germline clone (GLC) mutant NBs (90%, *n*=94; M) at the expense of basally localised protein. Miranda, red; γ-tubulin, green. Scale bar: 10 μm.

and Aurora A (Wang et al., 2006). We have shown that the efficient localisation of the adapter protein Miranda to the NB basal cortex requires the activity of the APC/C mitotic regulator. Mutations in several APC/C core subunits showed reduced cortically localised Miranda, with cytosolic accumulation of Miranda in an as yet unidentified pericentrosomal compartment. By contrast, apical complex formation was unaffected in these mutant NBs, showing that the APC/C acts downstream of or in parallel to the apical complex to ensure proper basal protein localisation. Furthermore, the basal localisation of PON/Numb were also unaffected by loss of APC/C activity, suggesting that Miranda itself might be a specific target for APC/C activity in mitotic NBs. This is further supported by the observation that the *ida* mutant phenotype can be partially rescued by specifically reducing Miranda protein levels.

The APC/C functions as an E3 ubiquitin ligase, and we have shown that Miranda is a ubiquitylated protein in both cultured cells and larval NBs. Our extensive attempts to demonstrate that Miranda ubiquitylation is APC/C-dependent have proved inconclusive. We therefore cannot rule out the possibility that the effects of loss of APC/C activity on Miranda localisation might be indirect. However, we have demonstrated that the phenotypes observed in APC/C mutants are recapitulated in the *miranda*^{RR127} allele encoding a C-terminal truncation of the protein. We have shown that not only are both Miranda localisation and ubiquitylation dependent on this region of protein, but that replacement of this domain with ubiquitin is able to restore wild-type protein localisation. Although the C-terminal domain of Miranda is clearly required for ubiquitylation and cortical localisation of the protein, the precise ubiquitylation sites in Miranda are as yet unknown and will be an interesting area for further study. We therefore speculate that the pericentrosomal accumulation of Miranda and the reduction of basally localised protein in APC/C mutants might reflect a loss of Miranda ubiquitylation. Recently, it has been shown that the ubiquitin moiety itself can function as a protein-protein interaction domain (Meyer et al., 2002; Sun and Chen, 2004). Ubiquitylation of Miranda could function as a signal to regulate its transport to the basal cell cortex perhaps by influencing its association with motor proteins that mediate basal protein targeting. Alternatively, ubiquitylation of Miranda could regulate the retention of Miranda at the basal cell cortex by influencing its association with anchoring or scaffolding molecules. Efficient localisation and/or retention of Miranda to the basal cortex clearly requires APC/C activity, but the presence of

Miranda protein at the basal cortex in APC/C mutants, albeit at a much reduced level compared with wild-type, indicates that other processes and molecules might also be involved.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/21/3781/DC1

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