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The Seckel syndrome and centrosomal protein Ninein localizes asymmetrically to stem cell centrosomes but is not required for normal development, behavior, or DNA damage response in Drosophila

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ABSTRACT Ninein (Nin) is a centrosomal protein whose gene is mutated in Seckel syndrome (SCLK, MIM 210600), an inherited recessive disease that results in primordial dwarfism, cognitive deficiencies, and increased sensitivity to genotoxic stress. Nin regulates neural stem cell self-renewal, interkinetic nuclear migration, and microtubule assembly in mammals. Nin is evolutionarily conserved, yet its role in cell division and development has not been investigated in a model organism. Here we characterize the single Nin orthologue in Drosophila. Drosophila Nin localizes to the periphery of the centrosome but not at centriolar structures as in mammals. However, Nin shares the property of its mammalian orthologue of promoting microtubule assembly. In neural and germline stem cells, Nin localizes asymmetrically to the younger (daughter) centrosome, yet it is not required for the asymmetric division of stem cells. In wing epithelia and muscle, Nin localizes to noncentrosomal microtubule-organizing centers. Surprisingly, loss of nin expression from a nin mutant does not significantly affect embryonic and brain development, fertility, or locomotor performance of mutant flies or their survival upon exposure to DNA-damaging agents. Although it is not essential, our data suggest that Nin plays a supportive role in centrosomal and extracentrosomal microtubule organization and asymmetric stem cell division.

INTRODUCTION Microcephalic primordial dwarfism (PD) is a spectrum of inherited recessive developmental disorders that cause fetal growth failure resulting in severe dwarfism, microcephaly, and cognitive deficiencies (Majewski and Goecke, 1982; Kringseisen and Jackson, 2011; Megraw et al., 2011; Chavali et al., 2014). The most prevalent PD disorders include Seckel syndrome, microcephalic osteodysplastic PD (MOPD) types I and II, and Meier–Gorlin syndrome. Mutations of at least 14 genes have been associated with PD disorders (Chavali et al., 2014). The genes identified for Seckel syndrome encode proteins that are fundamental to centrosome function (NIN, CEP63, CENPJ/CPAP/SAS-4, and CEP152; Al-Dosari et al., 2010;
Kalay et al., 2011; Sir et al., 2011; Dauber et al., 2012) and the DNA damage response (ATR, ATR-interacting protein [ATRIP], DNA2, and RBBP8/CTIP; O’Driscoll et al., 2003; Quist et al., 2011; Ogi et al., 2012; Shaheen et al., 2014). However, whether the functions of all of these genes are integrated into a common pathway responsible for Seckel syndrome is unclear (Arquint et al., 2014; Chavali et al., 2014; Antonczak et al., 2016).

The centrosome is the major microtubule-organizing center (MTOC) in most animal cells. It is composed of a pair of centrioles (a mother and its daughter), which organize a supramolecular protein assembly (Mennella et al., 2014; Woodruff et al., 2014), the pericentriolar material (PCM), where microtubule assembly and anchoring is regulated. During late mitosis, the pair of centrioles inherited by each cell mature, allowing them to assemble PCM and become centrosomes. Then, after centriole duplication in S phase, only the mother centriole is largely responsible for organizing the PCM at mitotic centrosomes (Wang et al., 2011). Although the centrosome is dispensable for proper cell division (Megraw et al., 1999; Khodjakov et al., 2000; Lecland et al., 2013) and even for most of Drosophila development (Megraw et al., 2001; Basto et al., 2006; Debec et al., 2010), mutations in the core centrosome machinery result in a spectrum of diseases that cause primordial dwarfsisms and impairment of brain development (microcephaly; Megraw et al., 2011; Chavali et al., 2015). Nin was recently identified as one of the genes that cause Seckel syndrome when mutated (Dauber et al., 2012). However, despite its importance to human health and mammalian brain development, the functions of Ninein (Nin) at the developmental, cellular, and molecular levels are not clearly defined.

In mammals, Nin is enriched around the centriole wall and at the subdistal appendages, structures present only on the mother centriole (Ou et al., 2002), which can anchor microtubules to the centrosome (Delgehyr et al., 2005). Nin and its paralogue in vertebrates, Ninein-like protein (Nlp), are γ-tubulin complex–associated proteins that regulate microtubule nucleation and anchoring at centrosomes and noncentrosomal MTOCs (Mogensen et al., 2000; Casenghi et al., 2003; Delgehyr et al., 2005).

In mouse embryonic neural progenitor cells, the older (mother) centrosome is inherited by the self-renewing stem cells at each asymmetric division (Wang et al., 2009). RNA interference (RNAi)–mediated Nin knockdown in embryonic mouse brains in utero disrupted asymmetric segregation of mother and daughter centrosomes and also reduced the number of radial glia progenitors in the developing neocortex of mice, indicating that Nin was required for maintaining asymmetric centrosome inheritance and suggesting that this regulates progenitor self-renewal (Wang et al., 2009). In zebrafish, knockdown of nin expression with morpholinos impairs growth and development of the midbrain–hindbrain boundary and formation of the anterior neuroectoderm (Dauber et al., 2012). Despite these loss-of-function studies on mammalian Nin using RNAi and morpholino approaches, there have been no mutant studies of Nin, and no whole-organism disease model has been established to study Seckel syndrome caused by disruption of Nin.

Here we identify the protein encoded by the Blastoderm-specific gene 25D (Bsg25D, hereafter referred to as ninein or nin) as the apparent sole homologue of the Ninein family in Drosophila. We present genetic, cell biological, and biochemical evidence that Nin shares key similarities with its mammalian counterpart but also some striking differences.

**RESULTS**

A single Nin-family orthologue in Drosophila

To identify putative members of the Nin family in Drosophila, we performed a sequence alignment using the National Center for Biotechnology Information Psi-BLAST program with the N-terminal region of human Nin as a query. This portion of Nin protein sequence contains two regions conserved in the Nin family in mammals, and it was shown to associate with components of the γ7-tubulin complex by immunoprecipitation (Casenghi et al., 2003; Delgehyr et al., 2005). In contrast to the rest of the Ninein primary sequence, the N-terminal domain is also not predicted to be coiled-coil and is therefore more likely to be conserved throughout evolution. This analysis identified the gene Bsg25D (CG14025) as the putative member of the ninein family in Drosophila (Figure 1). Subsequent phylogenetic analysis revealed that lower metazoan species possess a single nin ancestor gene that might have duplicated in the phylum Chordata. In addition to the apparent homology ascertained from sequence similarity, we also found Nin associated with other centrosome proteins (Gopalakrishnan et al., 2011).

Nin can assemble microtubule-organizing centers

To test whether Drosophila Nin shares the microtubule anchoring and nucleation function of vertebrate Nin, we expressed Nin–green fluorescent protein (GFP) in S2 cells, a Drosophila cell line of embryonic origin. For this and all experiments in which a nin transgene was expressed, the protein encoded by the nin-RB isoform was used (see Figure 6A later in this paper and Materials and Methods). In the majority of S2 cells, Nin accumulated in large cytoplasmic assemblies and occasionally clustered in the proximity of the plasma membrane. The Nin-GFP foci in S2 cells were sufficient to establish a partial reorganization of the microtubule cytoskeleton into a polarized microtubule array (Figure 2A and Supplemental Figure S1A). This is remarkable, considering that in interphase S2 cells, centrosomes normally do not act as major microtubule-organizing centers and microtubules are nucleated from many regions in the cytoplasm, including Golgi membrane (Rogers et al., 2008). Nin structures were not associated with Golgi markers, as shown by costaining with dGMAP, and do not appear to induce Golgi dispersion, in contrast to mammalian cells (Casenghi et al., 2005; Figure 2A). Nin colocalized partially with centrosomin (Cnn), a component of the PCM, but was more concentrated in the space surrounding the PCM (Figure 3, B and C).

To better understand the mechanism of microtubule organization by Nin, we observed individual microtubule nucleation events by labeling the plus end of growing microtubules with the plus end–tracking protein EB1 in S2 cells expressing EB1–GFP (Figure 2, B and C). Live-cell imaging experiments show an enrichment of plus ends of growing microtubules in the region where Nin is concentrated and apparent microtubule anchoring (Supplemental Videos S1–S4). Pairwise distance analysis of the points of emergence of EB1 comets shows that in the presence of Nin, microtubule nucleation sites cluster together when compared with wild-type cells expressing EB1–monomeric red fluorescent protein (mRFP) alone, which normally grow microtubules from many regions in the cytoplasm. The N-terminal conserved domain associates with γ-tubulin in Drosophila (Figure 2D), consistent with the ability of mammalian Nin to bind γ-tubulin complex components (Casenghi et al., 2003; Delgehyr et al., 2005). However, sites of microtubule clustering established by Nin overexpression in S2 cells are not enriched in γ-tubulin (unpublished data). Taken together, these experiments demonstrate that Drosophila Nin has the capacity to function similarly to vertebrate Nin as a regulator of microtubules at centrosomes.

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Nin is localized at the periphery of centrosomes and to noncentrosomal MTOCs

To examine the endogenous localization of Drosophila Nin, we generated and acquired antibodies that recognize different regions of Nin (Figure 1A) and used the antibodies to examine Nin localization in early cleavage-stage embryos. We selected this tissue because during early embryogenesis, centrosomes organize microtubules throughout the cell cycle, and previous studies suggested that Nin is highly expressed at this stage of development (Boyer et al., 1987). Antibody staining revealed that Nin localizes to the vicinity of centrosomes in blastoderm embryos and is enriched in pole cells, the precursor of germline cells (Figure 3, A and B), where it remains highly expressed in primordial germ cells late into embryogenesis (Supplemental Figure S2). We found that Nin localizes to the periphery of the centrosome, with only a partial overlap with the pericentriolar region labeled with antibodies against Cnn (Figure 3B). The Nin signal is higher at interphase centrosomes than at mitotic centrosomes (Figure 3B; see also Supplemental Figure S3), similar to the cell cycle dynamics reported for Nin in mammals (Mogensen et al., 2000; Casenghi et al., 2003). In addition to Nin localization by antibody staining, we constructed Nin-myc and Nin-GFP transgenic flies and examined their localization and dynamics in fixed and live embryos. The localization of Nin-myc was similar to endogenous, except that a higher signal could be seen at mitotic centrosomes (Figure 3C), likely because of overexpression. Live imaging by spinning-disk confocal microscopy of Drosophila early embryos expressing Nin-GFP shows that Nin is dynamically distributed at the centrosome periphery during the cell cycle, with enrichment at interphase centrosomes (Supplemental Figure S3 and Supplemental Videos S5 and S6). The localization and dynamics of Nin-GFP in live embryos are consistent with the distribution of endogenous Nin observed by antibody staining on fixed Drosophila embryos (Figure 3B).

Mammalian Nin was shown to localize to noncentrosomal MTOCs in specialized cell types (Mogensen et al., 2000). In Drosophila wing epithelial cells and myocytes, which organize microtubules from MTOCs at adherens junctions near the apical membrane and at perinuclear sites, respectively (Tassin et al., 1985; Mogensen et al., 1989; Bugnard et al., 2005), Nin was localized to these MTOCs (Figure 4). In wing epithelial cells, where previous reports showed that microtubules

**FIGURE 1:** Drosophila Nin (Bsg25D or CG14025) is the sole ortholog of mammalian Ninein and Ninein-like protein (Nlp). (A) The Ninein (Nin) family members share a highly conserved domain near the N-terminus that regulates microtubule assembly (shown as a green box), and multiple coiled-coil regions. Mammalian Nin has a centriole-targeting domain (red box) that is not conserved in Drosophila or in the mammalian paralog. The sequences corresponding to polypeptides used to raise antibodies are indicated. (B) Tree showing phylogenetic relationships among the Nin orthologues and paralogs. (C) Results of BLAST alignments show that Drosophila Nin has significant similarity to human Nin and Ninein-like protein (Nlp). Protein sequences conserved among Nin orthologues of metazoans are highlighted in red, and those conserved among Nlp orthologues are highlighted in blue. Note that Drosophila Nin shares residues with both Nin and Nlp (highlighted in green).
Drosophila stem cells. In Drosophila germ-line stem cells (GSCs) and neuroblasts (NBs), the centrosomes are segregated asymmetrically during cell division (Rusan and Peifer, 2007; Yamashita et al., 2007; Januschke et al., 2011, 2013). In male GSCs, the older, “mother” centrosome is retained in the GSC at each self-renewal, with the centrosome anchored at the apical membrane adjacent to the “hub,” the niche where the GSC resides (Yamashita et al., 2007). In the ovary, this asymmetry is inverted, and the younger, daughter centrosome is retained in the GSC (Salzmann et al., 2014). In NBs, the younger, daughter centrosome is retained in the self-renewed stem cell and retains PCM and MTOC activity in interphase, whereas the mother centrosome becomes inactivated until mitosis. This segregation pattern in NBs is opposite to what occurs in mouse neural progenitors, where the mother centrosome is retained by the self-renewed progenitors (Rusan and Peifer, 2007; Wang et al., 2009; Conduit and Raff, 2010; Januschke et al., 2011, 2013).

In larval brain NBs, we were unable to detect endogenous Nin localization to centrosomes with anti-Nin antibodies (unpublished data). However, in larval brain NBs expressing either Nin-myc (Figure 5A) or Nin-GFP (Supplemental Figure S1, B and C), Nin exhibited pericentrosomal localization, as revealed by largely nonoverlapping localization in close proximity to Cnn, similar to the pattern in embryos (Figure 3, B and C). Using Cnn to differentiate mother and daughter centrosomes, as Cnn is enriched at the daughter centrosome in NBs (Rusan and Peifer, 2007; Conduit and Raff, 2010; Januschke et al., 2011), we found that Nin preferentially accumulated at the younger, daughter centrosome rather than with the older, mother centrosome in NBs (Figure 5A). Nin localization at the daughter centrosome is cell cycle regulated in NBs, as Nin expression was only detected in interphase or early mitotic NBs and became undetectable at centrosomes by metaphase of mitosis. In male GSCs, expression of Nin-myc revealed that Nin localization was enriched preferentially at the daughter centrosome in this stem cell population too (Figure 5B). In contrast with Nin asymmetric localization in GSCs and NBs, Nin is symmetrically localized to centrosome pairs in embryos (Figure 3B) and ganglion mother cells in the developing brain (Supplemental Figure S1C), consistent with their symmetrical cell division characteristics.

Despite the provocative asymmetric localization observed with Nin transgene expression in NBs, we failed to detect endogenous Nin expression at larval NB centrosomes by immunofluorescence staining despite detection of Nin in lysates of whole brains by Western blotting (see later discussion). Whether this result reflects a lack of endogenous Nin expression in larval brain NBs or is due to insufficient sensitivity of our Nin antibodies is unclear. However, to

**FIGURE 2:** Nin organizes microtubule-nucleating centers when overexpressed in Drosophila S2 cells. (A) Images of S2 cells expressing Nin-GFP. Microtubules are labeled with antibodies against α-tubulin, and Golgi with antibodies against GMAP. See also Supplemental Figure S1A. Scale bar, 5 μm. (B) Images of EB1-mRFP microtubule plus-end tracks in S2 cells with expression of Nin-GFP (bottom) or without (top). See also Supplemental Videos S1–S4. (C) Pairwise distance of EB1 emerging comets. Pattern of MT nucleation sites measured by plotting the point of emergence of each EB1 particle and correlating it with emergence of its neighbors. (D) GST-Nin N-terminal 241 amino acid domain binds to γ-tubulin in S2 cell lysates.

organized from the adherens junctions near the apical membrane (Matis et al., 2014), we found that Nin was localized to a focus at the center of this MTOC (Figure 4C) and was not localized at centrosomes (Figure 4, A and B). This pattern was the same in wing disks from early third-instar larvae (unpublished data) or wandering stages (Figure 4). In muscle from third-instar larvae, Nin localized to the perinuclear MTOCs (Figure 4D). Thus Nin localizes to noncentrosomal MTOCs in Drosophila.

**Nin localizes with an asymmetric bias to daughter centrosomes when overexpressed in stem cells**

Given the requirement of Nin for the asymmetric segregation of centrosomes in mouse embryonic neural progenitor cells (Wang et al., 2009), we investigated the Nin localization pattern in...
nin transcripts (Figure 6, A and B, and Supplemental Figure S4). Because the start codons and a significant portion of the N-terminal coding regions of all nin isoforms are deleted, including the N-terminal MT-regulating domain (Figure 1A), nin1 appears to be a strong allele and likely a null.

To determine whether nin1 disrupts expression of Nin, we examined Nin protein by Western blot and Nin localization to centrosomes by immunofluorescence analysis (Figure 6, C and D). Whereas endogenous Nin was detected in embryos and larval brain lysates by Western blotting (Figure 6C) using antibodies raised against the N- or C-terminal regions of Nin (Figure 1A), Nin expression was absent in nin1 embryos and larval brains (Figure 6, C and D).

Nin is not essential for neuroblast asymmetric division and self-renewal or for adult locomotor performance

Although mutation of human NIN disrupts normal development, resulting in Seckel syndrome (Dauber et al., 2012), surprisingly, nin1 mutant flies are homozygous viable, appear to develop normally, are fertile, and have no overt morphological or behavioral phenotypes. Because nin knockdown by RNAi in mice showed that Nin is essential for embryonic neural progenitor asymmetric division and self-renewal (Wang et al., 2009), we investigated these functions in nin1 mutant flies. In contrast with its role in mice, we found that nin1 NBs did not lose asymmetric division characteristics during mitosis (Figure 7, A and B), as revealed by normal localization of the NB marker and basal polarity protein Miranda (Mira; Cabernard and Doe, 2009) and apparently normal centrosome asymmetry with regard to Cnn localization during the NB cell cycle. Moreover, spindle orientation with respect to NB polarity, which is disrupted in some centrosome protein mutants (Giansanti et al., 2001; Megraw et al., 2001; Singh et al., 2014), was normal (Figure 7B). Accordingly, there was no significant change in the number of Mira-positive NBs between wild-type and nin1 mutant larval central brains (Figure 7C and Supplemental Figure S5), indicating that Drosophila Nin, in contrast to mammalian Nin, is not essential for NB asymmetric division and self-renewal. We did not assess nin1 GSCs for proper asymmetric division.

Proper NB proliferation is essential to generate sufficient neurons to populate the nervous system. A properly functioning nervous system is essential for many neurological activities, including locomotor function. Moreover, Nin is required for cilium assembly in mammalian cells (Graser et al., 2007), and cilia are required for normal locomotor function in Drosophila (Eberl et al., 2000; Caldwell et al., 2003). Therefore we evaluated the locomotor performance of nin1 adult flies using a climbing assay and determined that nin1

examine a role for Nin in NB division and polarity, we generated a nin mutant allele and tested its function in NB asymmetric division.

nin1 is a deletion allele that disrupts nin expression

To study the functions of Nin, we sought a mutant allele of the nin gene by mobilizing a P element transposon located within the 5′ untranslated region of the first exon of the nin gene locus (Figure 6A). From the P element excision, we isolated one allele that deleted ∼3.5 kb of nin on the 3′ side of the P element, generating an allele that we designated nin1, and which is predicted to disrupt all of the

FIGURE 3: Nin is a pericentrosomal protein. (A) Relatively higher expression of endogenous Nin in the germline precursor (pole) cells in early embryos. Fixed wild-type embryos were stained with the C-terminal Nin antibody. See also Supplemental Figure S2. (B) Pericentrosomal localization of endogenous Nin in cleavage stage embryos. Shown are cycle 12–13 embryos and stage 14 (cellularization) stained with antibodies to the N-terminal region of Nin. Nin signal is highest in interphase, and relatively reduced in mitosis. (C) Pericentrosomal localization of Nin-myc in embryos. Fixed embryos expressing Nin-myc were stained with anti-myc for Nin expression (red), anti-Cnn for centrosome PCM (white) and 4′,6-diamidino-2-phenylindole (DAPI) for DNA (blue). Scale bar, 10 μm
We examined the role of Nin in early embryo development, in which centrosome function is essential for the early cleavage cycles (de Saint Phalle and Sullivan, 1998; Rothwell et al., 1998; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999; Kao and Megraw, 2009). In wild-type embryos, Nin is localized to the periphery of centrosomes but absent in nin\(^1\) mutant embryos (Figure 6D). Nin appears to be dispensable for embryo development, as nin\(^1\) mutant embryos had only a slightly reduced hatch rate compared with isogenic wild-type (w\(^{1118}\)) embryos (84.5 ± 1.0% vs. 87.8 ± 1.1%), an insignificant difference (p = 0.092; Figure 8A). Moreover, cleavage furrows and other aspects of cleavage such as spindle morphology and nuclear positioning appeared normal in nin\(^1\) mutant embryos (Figure 8B and unpublished data), establishing that loss of function of Nin does not overtly affect Drosophila embryo development.

Recent reports indicated that Nin associates with Rab11, a small G protein and regulator of endosomal vesicle trafficking (Guruharsha et al., 2011). This is consistent with the pericentrosomal localization of Nin, a pattern similar to that of Rab11, which localizes to vesicles at the periphery of centrosomes during early embryo development (Riggs et al., 2003). Because Rab11 was the only binding partner of Nin recovered in a mass spectrometry screen (Guruharsha et al., 2011), we sought to determine whether Nin and Rab11 have a genetic interaction. To this end, we examined the embryo hatch rate of nin\(^1\) Rab11 double mutants. Rab11\(^{138b}\) is a hypomorphic allele; homozygotes are viable, and females have reduced fertility due to maternal effect lethality. Double mutants with a combination of nin\(^1\) and Rab11\(^{138b}\) are also homozygous viable with reduced female fertility. The nin\(^1\) mutant decreased the hatch rate of Rab11\(^{138b}\) embryos from 66.9 ± 2.9 to 58.5 ± 0.5% (Figure 8A). The significance of this enhancement of Rab11 maternal effect lethality by nin is of low significance, however, with p close to 0.05. The mild genetic effect could be an additive effect rather than a significant genetic interaction between nin\(^1\) and Rab11\(^{138b}\) alleles. Rab11-deficient embryos from transheterozygotes bearing a combination of the amorphic Rab11\(^{207}\) allele and Rab11\(^{138b}\) showed a hatch rate of 8.5 ± 2.0%, consistent with previously published measurements (Jankovicic et al., 2001), and combining it with the nin\(^1\) homozygous mutation lowered the embryo hatch rate to 1.2 ± 1.0% (p = 0.029). This decrease in Rab11\(^ {207}\)/Rab11\(^{138b}\) embryo survival due to nin\(^1\) may again be an additive effect rather than a synergistic genetic interaction.

Costaining of embryos for Nin and Nuf, a partner of Rab11 that localizes to endosomes, showed that Nin mostly did not colocalize with Nuf (Supplemental Figure S6).

**Nin is not essential for embryo development**

We also evaluated Nin genetic interaction with two centrosome components—Cnn, a PCM protein, and Bld10, a centriolar protein. Mutations in bld10 are viable, yet mutant males are infertile, whereas females are fertile (Mottier-Pavie and Megraw, 2009; Carvalho-Santos et al., 2012; Roque et al., 2012), whereas cnn mutants are maternal effect lethal and male sterile (Li et al., 1998; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999). Double mutants of nin\(^1\) with cnn\(^{250n}\) or cnn\(^{42t}\) (both are cnn null alleles) did not overtly modify phenotypes of cnn alone, as observed by adult morphological features and viability. Similarly, double mutants with nin\(^1\) and bld10\(^{04199}\) or bld10\(^{01795}\) did not modify phenotypes of bld10 alone, as assessed by adult morphology, viability, and female fertility. Together, these results indicate that Nin does not interact genetically with some centrosome proteins.

**Loss of function of Nin does not affect DNA damage response**

Because mammalian Nin is implicated in Seckel syndrome (Dauber et al., 2012), we sought to determine whether the nin\(^1\) mutant has
Mei-41, are similarly sensitive to mutagenic agents like hydroxyurea (HU) or methyl methanesulfonate (MMS). Using a mutation in mei-41 (mei-41^{118}) as a positive control and w^{118} as a negative control, we found that, like w^{118}, nin^{1} mutant flies develop and survive similar to wild type when exposed to 80 μM HU or 0.1% MMS (unpublished data), both of which were lethal to the mei-41^{205} mutant, as shown previously (Banga et al., 1986). Therefore the nin^{1} mutant appears to have a normal DNA damage response.

Global overexpression of Nin is lethal
To evaluate the phenotype of Nin gain of function, we overexpressed Nin ubiquitously or in restricted tissues in Drosophila and compared it to the overexpression of other centrosomal proteins (Cnn, Bld10, Sas6, and Rab11). Ubiquitous overexpression of Nin-GFP in transgenic flies using Act-Gal4 and Tub-Gal4 drivers resulted in death during the early pupal stage, whereas flies that overexpressed Cnn, Bld10, Sas6, or Rab11 developed normally (Table 1). However, flies developed efficiently and were healthy when Nin was overexpressed only in NBs or throughout the entire nervous system using worniu-Gal4 or Elav-Gal4, respectively. These data indicate that some tissues or cells, but not those of the nervous system, are sensitive to Nin overexpression.

DISCUSSION
In this study, we identify and characterize the function and requirement of the sole member of the Ninein family in Drosophila from the cellular to the organismal level. The findings presented here highlight both the conserved roles and the key differences of Ninein in Drosophila melanogaster compared with mammalian species in which the majority of the previous studies have been conducted. We found that the disruption of nin does not overtly affect cell division, development, viability, fertility, or locomotor behavior in Drosophila. These results are surprising, given the conservation of Nin in eukaryotic evolution and its importance in vertebrates revealed by RNAi and morpholino studies. There is no apparent paralogue in flies to account for lack of phenotype, as phylogenetic analysis shows that, whereas many metazoans have two Nin-family paralogues (Ninein and Nlp), Drosophila possesses only one orthologue of Nin. However, a recent study in Caenorhabditis elegans showed that the ninein orthologue (NOCA-1) functions redundantly with Patronin (Wang et al., 2015), another MT minus end protein, raising the possibility that Drosophila Nin might also function in parallel with Patronin (Goodwin and Vale, 2010).

In vertebrates, Ninein is a component of the mother centriole and is enriched in subdistal appendages (Mogensen et al., 2000; Delgehyr et al., 2005), structures required for microtubule anchoring and ciliogenesis. In further contrast to vertebrate Nin, we find that Drosophila Nin localizes to the periphery of the centrosome and does not reside at centrioles. This difference in localization might be explained at least in part by the absence of mother centriole appendage structures in Drosophila centrioles. In addition, Drosophila Nin appears to lack the centriole-targeting domain found in vertebrate Nin (Figure 1A). The higher accumulation of Nin at interphase versus mitotic centrosomes in embryos correlates directly with the relative intensity of astral microtubules at embryonic cleavage-stage centrosomes, which are higher in interphase and lower in metaphase (Karr and Alberts, 1986), suggesting that Nin localization might depend on MTOC activity or may localize to MT minus ends together with Patronin, as recently shown in C. elegans (Wang et al., 2015).

Despite the differences in centrosomal localization, one aspect of Nin localization—its asymmetric localization in neural stem cells—suggests conservation of function with vertebrates with regard to

FIGURE 5: Nin localizes asymmetrically to daughter centrosomes in stem cells. (A) Nin-myc localization is asymmetric and enriched at daughter centrosomes in larval brain neuroblasts. Larval brain neuroblasts expressing Nin-myc were stained with anti-myc (red) to reveal Nin expression and localization. Cnn (white) labels centrosomes and their asymmetry (Cnn is enriched at daughter centrosomes), α-tubulin (green) for microtubules (MT), DAPI (blue) for DNA. See also Supplemental Figure S1B, C. M = mother centrosome, D = daughter centrosome. Scale bar, 10 μm. (B) Nin-myc localization at mother and daughter centrosomes in male germline stem cells (GSCs). Male GSCs expressing Nin-myc were stained with anti-myc for Nin expression, anti-FasIII to label the stem cell niche, anti-Cnn for centrosome PCM, and DAPI for DNA.
FIGURE 6: \(\text{n}in^1\) is a deletion allele that disrupts \(\text{n}in\) expression. (A) Schematic view of \(\text{Drosophila}\) \(\text{n}in\) locus, transcripts, \(\text{P}\) element insertion (G13518) and \(\text{n}in^1\) deletion. The \(\text{n}in^1\) deletion allele was generated by mobilizing the \(\text{P}\) element transposon, \(\text{Bsg25D}^{\text{G13518}}\), located in the 5’ UTR. The primer pairs used for PCR screening are indicated (arrows). The primer pairs in the boxed region were used to narrow down the region deleted in \(\text{n}in^1\). The dotted line section of the deletion represents the region where the 3’ breakpoint of the \(\text{n}in^1\) deletion resides: somewhere between the “e” and “F5” primer sites. The scale bar is 1 kb. (B) Single adult fly PCR analysis of \(\text{n}in^1\), which shows a deletion of ∼3.5 kb. The PCR results for the primer pairs in the boxed region are shown in Supplemental Figure S4. Control (Ctrl) represents the original \(\text{P}\) element insertion stock that was used to generate the \(\text{n}in^1\) deletion allele. Sequences for the primers are listed in Supplemental Table S1. (C) Western blot analysis of \(\text{n}in^1\) embryo and larval brain lysates using an antibody against the N-terminal region of \(\text{n}in\), and in embryo lysates also using a C-terminal antibody. The \(\text{Nin-myc}\) transgene shows endogenous and myc-tagged \(\text{Nin}\) bands, which are absent in \(\text{n}in^1\) lysates. \(\alpha\)-\(\text{tubulin}\) serves as loading control. (D) Immunofluorescence staining of wild-type and \(\text{n}in^1\) embryos. Fixed embryos were stained with antibodies against endogenous \(\text{Nin}\) N-terminal region, and with \(\text{Cnn}\) antibody to mark centrosomes. The inset shows magnified view of \(\text{Nin}\) and \(\text{Cnn}\) in embryos.

neural stem cell division and self-renewal in \(\text{Drosophila}\). However, whereas mouse \(\text{Nin}\) is localized preferentially to the mother centriole, it appears equally distributed between the centrosomes in neural progenitor cells (Wang et al., 2009). \(\text{Drosophila}\) \(\text{Nin}\), on the other hand, is localized preferentially to the younger, daughter centrosome and is barely detectable at the older, mother centrosome at the basal side of the NB, even at early mitosis, when PCD accumulates on the mother centrosome. This asymmetric localization of \(\text{Nin}\) in NBs is also recapitated in male germ line stem cells, where again \(\text{Nin}\) is enriched at the daughter centrosome and is expressed at relatively higher levels in the primordial germ cells (pole cells). The localization of \(\text{Nin}\) in both of these stem cells suggests a possible function in asymmetric cell division, which was not revealed upon depletion of \(\text{Nin}\) by the \(\text{n}in^1\) mutant.

The strong \(\text{n}in\) allele that we generated, \(\text{n}in^1\), disrupts \(\text{n}in\) expression. The mutant flies are viable and show no overt phenotypes. After carefully evaluating phenotypes in neural stem cells and embryos, we conclude that \(\text{Drosophila}\) \(\text{Nin}\) does not play an essential role in the nervous system or in early embryo development. Because human \(\text{NIN}\) is mutated in \(\text{SCHK}\) syndrome, which results in severe developmental defects and has etiological links to DNA damage response, it is further surprising that requirements for \(\text{Nin}\) in development and the DNA damage response were not conserved in \(\text{Drosophila}\). Taken together, our findings indicate that \(\text{Nin}\) plays a less critical role in flies than in \(\text{C. elegans}\) or vertebrate species.

On the basis of these findings, we speculate that \(\text{Nin}\) functions differently in \(\text{Drosophila}\) neuroblasts than in mammalian neural progenitors (Wang et al., 2009). Although no clear \(\text{n}in\) paralogues are present in \(\text{Drosophila}\), it remains possible that other protein(s) are functionally redundant with \(\text{Nin}\), masking any phenotypes in the \(\text{n}in^1\) mutant. Therefore, although \(\text{Nin}\) is not essential for \(\text{Drosophila}\) development, it may well have important functions that were not revealed by these experiments.

MATERIALS AND METHODS

\(\text{Drosophila}\) stocks

\(w^{118}\) was used as wild-type control. The \(\text{n}in^1\) deletion mutant is described later. The following stocks were obtained from the Bloomington \(\text{Drosophila}\) Stock Center with stock numbers indicated or from cited sources: \(\text{Bsg25D}^{\text{G13518}}\)/\(\text{CyO}\) (\(\text{n}in\) \(\text{P}\) element insertion allele; BL#28091), \(\text{Df(2)l}\)/\(\text{eXel}\) (BL#7497), \(\text{Rab11}^{\text{G13518}}\)/\(\text{TM3}\) (BL#4158), \(\text{Rab11}^{\text{D201}}\)/\(\text{TM3}\) (BL#12148), \(\text{cnn}^{\text{2501}}\)/\(\text{CyO}\), \(\text{K-R}\)\(\text{GAL4}^{\text{DC3}}\) UAS-\(\text{GFP}^{\text{DC3}}\), \(\text{cnn}^{\text{2542}}\)/\(\text{CyO}\), \(\text{K-R}\)\(\text{GAL4}^{\text{DC3}}\) UAS-\(\text{GFP}^{\text{DC3}}\) (Megraw et al., 1999), \(\text{bcd}^{\text{10}^{\text{G13518}}1}\)/\(\text{TM6}\) and \(\text{bcd}^{\text{10}^{\text{G13518}}5}/\text{TM6}\) (Mottier-Pavie and Megraw, 2009), and \(\text{mei-4}^{\text{D25}}\) (BL#4236).
FIGURE 7: Nin is not essential for neuroblast asymmetric division and self-renewal, or for normal locomotor function. (A) Representative images showing the normal asymmetric division of nin1 larval brain NBs at the indicated stages of the cell cycle. Mira staining (red) is a NB basal marker of cell polarity, phospho-histone 3 (PH3, blue) for mitotic cells, Cnn (green) for centrosome PCM. (B) Polarity and spindle orientation are normal in nin1 NBs. Left: NBs were stained with anti-Mira (red), anti-aPKC (green) for basal and apical polarity, respectively, and anti-a-tubulin (blue) for microtubules. Right: the percent spindles oriented along the polarity axis, in 15 degree increments, is quantified. (C) Quantification of central brain Mira-positive nuclei shows normal number of NBs in nin1 larval brains. See Supplemental Figure S6 for representative staining. (D) Climbing (negative geotaxis) assay shows normal locomotor performance for nin1 adult flies. Error bars in C and D indicate SE of the means (SEM).

Generation of UAS-Nin-GFP and UASP-Nin-myc transgenes

The Nin coding sequence corresponding to the nin-RB isoform (Figure 6A) was amplified by PCR from the LD21844 cDNA clone obtained from the Drosophila Genetics Resource Center (DGRC). The sequence, including the ATG codon and encompassing the entire open reading frame (ORF) up to the last codon but excluding the stop codon, was amplified by PCR using ninRB-F1 and ninRB-R1 (Supplemental Table S1) and cloned into the pENTR vector (Life Technologies, Carlsbad, CA). The ORF was transferred by the Gateway system (Life Technologies) into several expression vectors (Teresa Murphy [Carnegie Institution for Science] and DGRC), which were modified by the attB sequence: pTWG-attB and pPWM-attB vectors were constructed by cloning a 368–base pair fragment containing the attB sequence PCR amplified from pVAU1M1 (base pairs 2567–2935) using primers attB-F1 and attB-R1 (Supplemental Table S1) into the AattII restriction site of pTWG and pPWM (at the 1989–base pair position in both vectors), respectively (Chen and Megraw, 2014). Transgenic flies were generated by Genetvision. These nin transgenes were inserted at a PhiC31 landing site 51D9.

Phylogenetic analysis

The putative non–coiled-coil region of the Nin-PB isoform (amino acids 1–341) was used as a bait to run three rounds of PSI BLAST. Most genes homologous to NInein were identified after only one round of PSI BLAST, whereas the C. elegans homologue was identified after two rounds, suggesting a high degree of sequence divergence in this species. Every identified gene was then reversed BLASTed against the Drosophila genome. No clear homologue of Nin in was found in the yeast model organisms Saccharomyces cerevisiae and Schizosaccharomyces pombe. After identification of several genes with homology to Nin in different species, the N-terminal 1– to 341–amino acid sequence was used to run a sequence alignment by using the program MAFFT. The phylogenetic tree was built with the neighbor-joining method using C. elegans sequence as outgroup; bootstrap analysis was performed using the program MacVector.

Immunofluorescence and live-cell imaging of S2 cells

The nin-RB ORF was cloned into the pAWG and pMT/V5-HIS expression vectors for expression in Drosophila Kc167 cells and S2 cells, respectively. Transfections were performed with Lipofectamine 2000 (Life Technologies) or Effectene (Qiagen, Hilden, Germany). Ectopic expression of fluorescently tagged Nin in S2 cells was driven by addition of CuSO4 at a final concentration of 300 μM. Immunostaining was performed as described (Kao and Megraw, 2004; Mennella et al., 2012). Live-cell imaging on S2 cells and Drosophila embryos was performed as previously described (Rogers et al., 2002). Images were acquired with a Zeiss Axiovert 200M equipped with a 100×/1.45 numerical aperture objective and an electron-multiply charged-coupled device camera (C9100-13; Hamamatsu Photonics, Japan). The 488-nm line of an argon laser or the 561-nm line of a krypton laser was used for illumination, attached to a spinning-disk confocal scan head (CSU10; Yokogawa; obtained from Solamere).

Analysis of microtubule dynamics in Drosophila cells

Drosophila S2 cells were cotransfected with expression plasmids (pMT/V5His) encoding EB1-GFP or EB1-RFP (Mennella et al., 2005) plus Nin-GFP. At 48 h after transfection, expression was induced by addition of CuSO4. Cells were subsequently plated on #1.5 glass-bottom MatTek dishes for 4 h and imaged by spinning-disk confocal fluorescence microscopy. The pixel coordinates of newly emerging EB1 comets were obtained by analysis of the maximum intensity projections of the recorded time-lapse video. The pairwise distance between each of the individual new EB1 comets in the time lapse was determined with an Excel macro. Statistical analysis was performed with Kaleidagraph software.
FIGURE 8: Nin is not essential for embryo development and nin is not interact genetically with Rab11. (A) nin embryos hatched at a slightly decreased rate (84.5 ± 1.0%) compared with wild type (87.8 ± 1.1%). Homozygous Rab11 showed a significantly reduced hatching rate of 66.9 ± 2.9% compared with wild-type, while addition of nin decreased the hatching rate further to 58.5 ± 0.5%. Only 8.5 ± 2.0% of transheterozygous Rab11/Rab11 hatched. However, in combination with nin the hatching rate reduced to 1.2 ± 1.0%. Three independent experiments were performed. 1000 embryos each were assayed for each of the remaining genotypes. Error bars indicate SEM. (B) Representative images showing normal cleavage furrow formation in nin embryos. Embryos were stained with anti-a-tubulin (green), phospho-tyrosine (red, marker for cleavage furrow), and DAPI (blue).

Generation of nin deletion allele

A collection of ~300 potential nin deletion alleles was generated by mobilizing a P element transposon located within the S′ UTR of the nin gene in the Bsg25D allele (Figure 6A) by crossing it through the germ line of females carrying P transposase (CyO, p2A3; BL#6394). All potential alleles were balanced with CyO, P(GAL4-Kr), and P(UAS-GFP) (BL#5194). We isolated both w– and w+ flies from these crosses. To screen for deletions in the nin gene caused by imprecise excision of the P element, adult flies or third instar larvae homozygous or hemizygous over Df(2L)Exel6011 (BL#7497) were analyzed by PCR using primers indicated in Figure 6A and listed in Supplemental Table S1 using the single-fly PCR method (Gloor and Engels, 1992). Initial screening for deletion alleles was performed with primer pairs proximal to the transposon insertion site. Only one allele was found that lacked this sequence and failed to produce a PCR product; we named this allele nin. Subsequent PCR analysis was performed with primer pairs extending into the 3′ end of nin, showing that nin has an –3.5-kb deletion starting from the N-terminus and removing most of the coding sequence of all isoforms of the nin gene (Figure 6 and Supplemental Figure S4). Therefore nin is likely a null allele. The exact 3′ breakpoint of the deletion was not mapped at the nucleotide level but to within a 300-base pair region. A remnant of the original transposon remains at the 5′ end of the nin allele and retains the w minigene. Thus nin is tightly linked with a white minigene and has an orange eye color.

The nin allele was backcrossed with w1118 for six generations to remove other potential lesions on the chromosome and isogenize it with the control w1118 stock. The resulting potential deletion mutant could be maintained as a homozygous stock.

Antibodies

The following antibodies were used: mouse anti-a-tubulin (DM1A; 1:1,000 for indirect immunofluorescence staining [IF], 1:10,000 for immunoblotting [IB]; Sigma-Aldrich, St. Louis, MO), rat monoclonal anti-a-tubulin (YL1/2; 1:1,000 for IF; Thermo Fisher, Waltham, MA), mouse monoclonal anti-my (9B11; 1:2,000 for IF; 1:20,000 for IB; Cell Signaling Technology, Danvers, MA), rabbit or guinea pig anti-Cnn (1:1,000 for IF; Zhang and Megraw, 2007), guinea pig anti-Nin N-terminus (1:100 for IF; Zhang and Megraw, 2007), guinea pig anti-Nin C-terminus (see later discussion; 1:2,000 for IB), rat anti-Mira (1:100 for IF; a gift from Chris Doe, Institute of Molecular Biology, University of Oregon, Eugene, OR), rabbit anti-aPKC (C-20; 1:100 for IF; Santa Cruz Biotechnology, Dallas, TX), rat anti-phospho-histone H3 (1:1,000 for IF; Upstate EMD Millipore, Darmstadt, Germany), mouse anti-phosphotyrosine (1:500 for IF; Santa Cruz Biotechnology), rabbit anti-Nuf (1:500 for IF; GTU-88; Sigma-Aldrich), anti-GM130

| Expression pattern     | Cross               | F1 Phenotype     |
|------------------------|---------------------|-----------------|
| Throughout nervous system | Elav-GAL4 × UAS-Nin-GFP | Healthy and fertile |
| Neuroblast-specific    | Worini-GAL4 × UAS-Nin-GFP | Healthy and fertile |
| Global                 | Tub-GAL4/TM68 × UAS-Nin-GFP | Early pupal lethal |
|                        | Tub-GAL4/TM68 × UAS-GFP-Cnn | Healthy and fertile |
|                        | Tub-GAL4/TM68 × UAS-Blal0-GFP | Healthy and fertile |
|                        | Tub-GAL4/TM68 × UAS-Sas6-GFP | Healthy and fertile |
|                        | Tub-GAL4/TM68 × UAS-Rab11-YFP | Healthy and fertile |

TABLE 1: Global Nin overexpression is lethal.
(1:1000 for IF; Abcam, Cambridge, United Kingdom), and guinea pig anti-Asterless (1:1000 for IF; a gift from Nasser Rusan, Cell Biology and Physiology Center, National Institutes of Health, Bethesda, MD). Anti-Dlg antibodies (1:100 for IF) were obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

**Generation of rabbit anti-Nin C-terminus antibodies**

Sequences corresponding to amino acids 829–1026 of Nin-PB were cloned into pDEST-17 (Invitrogen) for expression of a 6xHis-tagged protein in *E. coli* strain BL21(DE3)pLysE. Nin protein was isolated from *E. coli* in inclusion bodies, dissolved in 6 M guanidine hydrochloride, centrifuged at 12,000 × g for 20 min, and purified by immobilized metal affinity chromatography with Ni²⁺-charged Che- lating Sepharose Fast Flow (GE Healthcare, Little Chalfont, United Kingdom). Purified protein was dialyzed against phosphate-buffered saline (PBS), and 3 mg purified by preparative minigel using 10% SDS–PAGE. The protein band was excised from the gel, and antibodies were raised in rabbits by Cocalico Biologicals. This antibody was used for Western blotting. A second antibody, used for immunostaining, was generated by immunizing rabbits (Covance, Princeton, NJ) with a synthetic peptide corresponding to amino acids 1074–1091 of Nin-PB. Antibodies were affinity-purified by peptide affinity chromatography using sulfolink resin (Pierce, Thermo Fisher).

**Western blotting**

6 h or overnight embryos were lysed in 2×SDS–PAGE loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.02% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol). Third instar wandering larval brains (n = 10) were dissected and lysed in 20 μl of 2×SDS–PAGE loading buffer. Boiled embryo and larval brain lysates were resolved by 7.5% SDS–PAGE and then transferred to nitrocellulose membranes (Santa Cruz) using a semidy transfer system (Bio-Rad, Hercules, CA). After blocking with 5% nonfat milk in 1×TBS for 1 h at room temperature, membranes were probed with primary antibodies diluted in 1×TBS containing 0.1% Tween and 2.5% nonfat milk overnight at 4°C. The membrane was then washed with 1×TBS containing 0.1% Tween three times for 5 min each. The washed membrane was incubated with secondary antibodies conjugated with IRDye-800CW or IRDye-680LT (1:20,000) for 1 h at room temperature. Signal was detected with an Odyssey Infrared Imaging system (LI-COR Bioscience, Lincoln, NE), followed by image processing in Adobe Photoshop CS4.

**Immunostaining of *Drosophila* tissues**

Nin imaging was performed using anti-Nin antibodies (either the N-term guinea pig one or one of the C-terminal rabbit ones; see above), unless otherwise indicated on the figures and in the figure legends. Larval brain neuroblast staining was performed as previously described (Kao and Megraw, 2009). Briefly, brains from third instar wandering larvae were dissected in PEM (100 mM PIPES, pH 6.9, 1 mM EGTA, and 2 mM MgSO₄) and transferred to a 4 μl drop of PEM on a clean slide, which was subsequently covered with a 22 × 22 mm siliconized coverslip containing 1 μl of 18.5% formaldehyde in PEM, allowing the weight of the coverslip to flatten the larval brains (two per slide) for 30 s. The slide was then immersed in liquid nitrogen for snap freezing. The coverslip was flipped off using a razor blade, and the glass slide with the brain attached was immediately fixed in −20°C methanol for 10 min, followed by a rinse in PBS. A hydrophobic ring is drawn around the brain tissue using a Super Pap Pen (ImmunoTech, Monrovia, CA), and 50 μl of primary or secondary antibodies solution (diluted in PBS containing 5% bovine serum albumin (BSA) and 0.1% saponin) was pipetted over the brain and contained within the ring. The slides were incubated in a humid chamber at room temperature for 1–2 h.

To examine polarity protein localization in larval brain neuroblasts and to assess neuroblast number and spindle orientation in whole larval brains, whole-mount larval brain staining was performed as previously described (Singh et al., 2014). Third instar wandering larval brains were dissected in PBS and fixed for 20 min in 4% paraformaldehyde in PBS. The fixed larval brains were washed twice with PBS, followed by blocking with PBSBT (1×PBS, 0.1% Triton X-100, and 1% BSA) for 1 h and incubated with primary and secondary antibodies in PBSBT.

Embryo collection, fixation, and staining were performed as previously described (Megraw et al., 1999). Fixed embryos were blocked 1 h with PBSBT and incubated with primary and secondary antibodies in PBSBT. Fixed and stained embryos were equilibrated in 80% glycerol in PBS, and were mounted in 25 μl of this mounting medium and stored at 4°C.

Whole-mount wing disk staining was similar to the larval brain staining as above described. Larval muscle was fixed and stained as described (Januschke et al., 2006). Briefly, third instar larvae were filed out and gutted, then the carcasses were extracted with 0.5% Triton X-100 prior to fixation in ice-cold methanol for 20 min. Following rehydration, muscle files were blocked for 1 h with PBSBT and incubated with primary and secondary antibodies in PBSBT. Fixed and stained files were equilibrated in 80% glycerol in PBS, and were mounted in this mounting medium and stored at 4°C.

Samples were imaged using a Nikon A1 laser scanning confocal microscope (Nikon, Japan) using a 60×1.49NA oil immersion objective, or on a Leica SP5 laser scanning confocal microscope using a 40×/1.4NA or a 63×/1.4NA oil immersion objective. Images were captured with a spacing of 0.5 μm between z-sections. All images are maximum intensity projections of z stacks, processed using the Nikon NIS-Elements software or the Leica SP5 software and Adobe Photoshop CS5.

**Identification of daughter and mother centrosome in neuroblasts**

In fixed samples, neuroblasts were generally identified as Mira-positive, or cells with >10 μm in diameter. The volume and fluorescence intensity of Cnn at centrosomes was used to distinguish mother and daughter centrosomes: the centrosome with larger diameter and higher fluorescence intensity were defined as the daughter centrosome (Rusan and Peifer, 2007; Januschke et al., 2011; Januschke et al., 2013). In telophase neuroblasts, we could readily distinguish daughter from mother centrosome because the daughter centrosome segregates with the neuroblast, which has a larger size relative to the ganglion mother cell, where the mother centrosome segregates.

**Analysis of mitotic spindle orientation and measurement of neuroblast number in larval brain**

To assess mitotic spindle orientation of neuroblasts in fixed larval brains, we plotted the angle between the mitotic spindle axis using tubulin staining or Cnn staining, with a tangential line of Mira crescent using Image J (NIH). For measurement of brain neuroblasts in wandering stage larvae, we didn’t include either the type II neuroblasts in the dorsoposterior brain that have more complex lineages or the optic lobe neuroblasts (Cabenard and Doe, 2009). The Mira positive neuroblasts were counted. The counts from the central
brain region and from the whole brain were quantified in Figure 7 and Supplemental Figure S6, respectively. The number of neuroblasts is shown as mean ± SEM from eight larval brains.

**Locomotor ability analysis of adult fly**

Fly locomotor performance assays were performed as previously described (Ali et al., 2011). Briefly, 10 well-fed male flies aged 6–8 d were placed into an empty chamber consisting of two conjoined plastic vials. Flies were gently tapped down to the bottom of vial and then given 10 s to climb a vertical 8 cm distance on the vial. The number of flies that successfully climbed 8 cm distance was recorded. The same set of flies were tested following a 1 min rest: this was repeated five times. The climbing index was calculated by the percent of flies passing the 8 cm mark and calculated as the mean ± SEM from at least five independent sets of 10 flies for each genotype.

**Analysis of embryo hatch rate**

nin1 mutant embryos were collected from nin1 homozygous females after crossing nin1 homozygous males and females. Rab11-deficient embryos were collected either from homozygous females bearing the hypomorphic allele Rab11J2D1 or from trans-heterozygous females bearing a combination of the amorphic Rab11J2B/ and hypomorphic Rab11J2D1 alleles (Jankovics et al., 2001). Double mutants bearing a combination of nin1 and Rab11J2B/alleles, or a combination of nin1 and Rab11J2D1 alleles were also tested.

Embryo hatching rate was determined with a method modified from (Kao and Megraw, 2009). Briefly, to count the hatching rate of embryos, 250 embryos for each genotype were lined up on apple juice/agar plates. After 2 d of incubation at 25°C, hatched and unhatched embryos were counted. Data shown were from at least three independent assays with a total number of at least 750 embryos from each genotype. Student's t-test was used to analyze the significance, and hatching differences were considered statistically significant when p < 0.05.

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