Loss of function of the *Drosophila* Ninein-related centrosomal protein Bsg25D causes mitotic defects and impairs embryonic development

Michelle Kowanda¹, Julie Bergalet², Michal Wieczorek¹, Gary Brouhard¹, Éric Lécuyer²,³,⁴ and Paul Lasko¹,*

**ABSTRACT**

The centrosome-associated proteins Ninein (Nin) and Ninein-like protein (Nlp) play significant roles in microtubule stability, nucleation and anchoring at the centrosome in mammalian cells. Here, we investigate *Blastoderm specific gene 25D (Bsg25D)*, which encodes the only *Drosophila* protein that is closely related to Nin and Nlp. In early embryos, we find that Bsg25D mRNA and Bsg25D protein are closely associated with centrosomes and astral microtubules. We show that sequences within the coding region and 3′UTR of Bsg25D mRNAs are important for proper localization of this transcript in oogenesis and embryogenesis. Ectopic expression of eGFP-Bsg25D from an unlocalized mRNA disrupts microtubule polarity in mid-oogenesis and compromises the distribution of the axis polarity determinant Gurken. Using total internal reflection fluorescence microscopy, we show that an N-terminal fragment of Bsg25D can bind microtubules in vitro and can move along them, predominantly toward minus-ends. While flies homozygous for a Bsg25D null mutation are viable and fertile, 70% of embryos lacking maternal and zygotic Bsg25D do not hatch and exhibit chromosome segregation defects, as well as detachment of centrosomes from mitotic spindles. We conclude that Bsg25D is a centrosomal protein that, while dispensable for viability, nevertheless helps ensure the integrity of mitotic divisions in *Drosophila*.

**KEY WORDS:** RNA localization, Centrosome, Dynein, Pole plasm

**INTRODUCTION**

Establishment of embryonic patterning in *Drosophila melanogaster* requires localized translation of numerous maternally deposited mRNAs in specific regions of the embryo during the initial nuclear divisions in the syncytial stage of embryogenesis (Lasko, 2012). *Drosophila* primordial germ cells, often called pole cells, are specified by localized posterior determinants, many of which are translated from mRNAs that accumulate in the posterior pole plasm of the oocyte and early embryo. At least 11 mRNAs known to be involved in pole cell development and/or embryonic patterning transiently accumulate in a perinuclear pattern around the pole cell nuclei during nuclear division 9 in embryogenesis, namely germ cell-less (Jongens et al., 1992), polar granule component (Hanyu-Nakamura et al., 2008), nanos (nos) (Wang and Lehmann, 1991), spire (Dahlgaard et al., 2007), Tao (Sato et al., 2007), arrest (Parisi et al., 2001), exuperantia (Winslow et al., 1988), oo18 RNA-Binding Protein (Lantz et al., 1994), tramtrack (Read et al., 1992), cyclin B (Kadyrova et al., 2007), and pumilio (Asaoka-Taguchi et al., 1999; Lécuyer et al., 2007). One of these mRNAs, nos, is first anchored to the posterior actin cytoskeleton, and then transported to the migrating posterior nuclei by the motor protein Dynein along astral microtubules (Lerit and Gavis, 2011). Vasa protein (Vas), a DEAD-box helicase essential for germ cell specification, localizes in the same pattern (Lerit and Gavis, 2011). It is assumed that other mRNAs with the same distribution pattern localize through a similar mechanism, although this has not been directly investigated.

In the early *Drosophila* embryo the first ten rounds of nuclear divisions are synchronous and are not coupled to cytokinesis (Foe and Alberts, 1983). During this period, nuclei migrate toward the periphery of the embryo. The next three rounds of nuclear division remain synchronous and uncoupled to cell divisions, except at the posterior pole of the embryo, where nuclei migrate through the germ plasm, slow their divisions and become incorporated within pole cells, the first distinctive cells to form in the embryo. Centrosomes that associate with the nuclei that migrate to the posterior trigger the release of germ plasm components, such as nos and Vas, from the embryonic posterior cortex, enabling Dynein-dependent transport into the pole cells as they form (Lerit and Gavis, 2011). This suggests that germ cell specification might be particularly sensitive to the activities of centrosome-associated proteins. Consistent with this, a role for Neur14, a centrosome-associated protein, in germ cell specification and integrity has recently been revealed (Jones and Macdonald, 2015).

A centrosome typically consists of a pair of centrioles surrounded by pericentriolar material, and it is from this structure that spindle and astral microtubules emanate (Azimzadeh and Bornens, 2007). Centrioles contain nine triplets of microtubules with proximal and distal ends (Vorobjev and Nadezhdina, 1987). The pericentriolar material is a highly organized structure (Fu and Glover, 2012; Lawo et al., 2012). Stringent control of the centrosome and centrioles is vital since abnormalities in spindle pole function can lead to genomic instability. Centrosomes in syncytial *Drosophila* embryos differ in composition from those of other animals; they are considered immature because they are shorter and have no clear difference between their proximal and distal ends (Gonzalez et al., 1998). In addition, centriole duplication occurs after centrosome separation (Callaini and Riparbelli, 1990). This is unlike in mammalian cells where duplication takes place prior to centrosome division (Callaini et al., 1997). Despite these
and zygotic expression of Bsg25D mRNA and subsequent embryonic patterning. Finally, we find that maternal Bsg25D is produced loss-of-function mutants and used them to investigate its functions during oogenesis and early embryogenesis. We show that Bsg25D mRNA is both maternally contributed during oogenesis and zygotically expressed in syncytial blastoderm stage embryos (Iampietro et al., 2014). However, recent results with more sensitive techniques have shown that, while Bsg25D is most abundantly expressed in early embryos, it is also expressed during many developmental stages (Roy et al., 2010; Wasbrugh et al., 2010; BDGP in situ homepage: http://insitu.fruitfly.org/cgi-bin/ex/insitu.pl). Bsg25D mRNA is both maternally contributed during oogenesis and zygotically expressed in syncytial blastoderm stage embryos (Lécuyer et al., 2007; Tomancak et al., 2007). This transcript is localized to the posterior pole plasm in early embryogenesis and exhibits a prominent perinuclear pattern around the pole cell nuclei during nuclear division 9, similar to nos and the other mRNAs discussed above, as well as peri-centrosomal localization in the somatic region of the embryo (Iampietro et al., 2014).

To explore the cellular and developmental roles of Bsg25D, we produced loss-of-function mutants and used them to investigate its functions during oogenesis and early embryogenesis. We show that Bsg25D protein and mRNA co-localize to centrosomes and microtubules in vivo and that a purified form of Bsg25D protein can bind to microtubules in vitro. Furthermore, the localization of Bsg25D in oogenesis and embryogenesis is dictated by separable localization elements within the coding region and 3′ UTR, while mislocalization of Bsg25D in oocytes affects microtubule polarity and subsequent embryonic patterning. Finally, we find that maternal and zygotic expression of Bsg25D is important for full embryonic viability and that mutant embryos frequently exhibit mitotic divisions prior to the midblastula transition (MBT) in embryogenesis.

**RESULTS**

**Localization of Bsg25D mRNA and protein is dynamic in oogenesis**

We first sought to characterize the distribution of Bsg25D mRNA and protein in oogenesis and early embryogenesis as a means of identifying possible sites where its function is required. In situ hybridization experiments indicated that Bsg25D mRNA is expressed throughout oogenesis. Like many other mRNAs that are ultimately destined for the posterior pole plasm, Bsg25D accumulates in the oocyte of stage 2-7 egg chambers. Unlike these others, however, Bsg25D mRNA becomes most concentrated at both the anterior and posterior poles of the oocyte from stage 10 onward (Fig. 1A-D). Immunohistochemical experiments using an antisera that recognizes Bsg25D (Iampietro et al., 2014) revealed that its protein expression largely mirrored that of its mRNA until stage 10, when anterior accumulation of the protein is not as apparent as for the mRNA (Fig. 1E-H).

In situ hybridization experiments confirmed previous evidence indicating that Bsg25D mRNA is enriched at the posterior of the embryo in its earliest stages of development, and then accumulates in the pole buds and pole cells as they form (Fig. 1I-L). Localization of Bsg25D mRNA to the posterior pole plasm is not absolute and some is apparent in somatic regions of the embryo as well, where it accumulates in two puncta on opposite sides of each nucleus (Iampietro et al., 2014; Lécuyer et al., 2007) (Fig. 1J). Immunohistochemical staining showed that Bsg25D protein is distributed in a similar pattern (Fig. 1M-P). To determine the relationship between Bsg25D puncta and centrosomes, we carried out double labeling experiments with antisera recognizing Bsg25D and the expression of centrosomal component Centrosomin fused to GFP (Cnn-GFP). We observed that Bsg25D and Cnn-GFP foci were closely associated, and that the foci of Bsg25D staining are generally larger than those of Cnn-GFP (Fig. 1Q). In many cases the Cnn signal was completely enveloped by the Bsg25D signal. Using a similar approach we also observed colocalization between Bsg25D and γ-tubulin throughout mitosis, but not with α-tubulin (Fig. S1). These results indicate that Bsg25D is a component of Drosophila centrosomes, or associates closely with them. Remarkably, Bsg25D mRNA also co-localized with centrosomes, both in posterior pole cells and in the somatic region of the embryo (Fig. 1Q,R). The accumulation of both Bsg25D mRNA and Bsg25D protein at centrosomes suggests that Bsg25D mRNA is translated locally there.

**Generation of Bsg25D mutant alleles**

To investigate Bsg25D function, we next used the ends-out gene targeting method (Maggert et al., 2008) to produce premature-termination (Bsg25D\(^\gamma\), first 353 amino acids) and null (Bsg25D\(^{Null}\)) alleles of Bsg25D (Fig. 2B). Bsg25D, like Nin and Nlp, has numerous coiled-coil domains (Fig. 2A). It also contains a predicted Smc chromosome segregation ATPase domain (Marchler-Bauer et al., 2015) that is also found in Nin, but not in Nlp (Fig. 2A). Nin and Nlp also contain predicted EF-hand domain pairs in their N-terminal regions (Fig. 2A). While substantial sequence similarity between Bsg25D and these proteins is present in their N-termini, some of the conserved residues of EF-hands are absent in Bsg25D. Based on these relationships we consider Bsg25D to be a closer orthologue to Nin than to Nlp. Bsg25D\(^{\gamma}\) lacks the Smc domain and all but one of the coiled domains (Fig. 2A-C). However, a similar N-terminal fragment of mouse Nin was found to co-purify, in co-immunoprecipitation and pull-down experiments, with γ-tubulin containing complexes (Delgehyr et al., 2005). The Bsg25D\(^{Null}\) allele does not detectably express Bsg25D protein at all (Fig. 2B,C).

Bsg25D mRNA localization involves elements within both its coding region and its 3′ UTR

We next aimed to identify potential localization elements in Bsg25D mRNA and to investigate whether localization of the mRNA is relevant to its in vivo function, we generated a series of transgenic fly lines (Fig. 3A). The genotypes of these flies were confirmed through PCR, northern blot, and immunostaining (Fig. S2A-D). UASp-eGFP-Bsg25D\(^{-CR_3′UTR}\) (CR_3′UTR) flies expressed the full-length coding region of Bsg25D (isofrom RB) along with the 3′ UTR, UASp-eGFP-Bsg25D\(^{-CR}\) (CR) expressed the full-length coding region but lacked the 3′ UTR, and UASp-eGFP-Bsg25D\(^{-3′UTR}\) (3′UTR) expressed only the 3′ UTR. UASp-eGFP (GFP) flies, expressing eGFP alone, served as a negative control.

Using these transgenic lines, we conducted in situ hybridization experiments with an eGFP probe to examine the distribution of transgenic eGFP-Bsg25D mRNAs in oocytes lacking endogenous Bsg25D. As expected, eGFP was uniformly distributed in the oocytes of Bsg25D\(^{Null}/\Delta\) females (Fig. 3B). When the full-length eGFP-Bsg25D transgene containing the 3′ UTR was expressed...
(Bsg25D\textsuperscript{Null}/Df; CR\textsubscript{3}'\textsuperscript{UTR}/NGV), eGFP distribution faithfully reproduced the pattern of endogenous Bsg25D (Fig. 3C, compare with Fig. 1D). In contrast, eGFP-Bsg25D mRNA containing the coding region alone (Bsg25D\textsuperscript{Null}/Df; CR/NGV) was concentrated at the anterior of the oocyte and did not accumulate at the oocyte posterior at stage 10a (Fig. 3D). Conversely, transgenic flies expressing only the eGFP-Bsg25D 3' UTR (Bsg25D\textsuperscript{Null}/Df; 3'UTR/NGV) precociously localized eGFP-Bsg25D RNA to the posterior at early stage 10 (Fig. 3E-G). Similar localization patterns are observed when these transgenes are expressed in a wild-type background (Fig. S2E-H).

Next, we examined the distribution of these transgenic mRNAs in otherwise wild-type embryos. In embryos from NGV; GFP mothers, eGFP is found generally in the cytoplasm (Fig. 3H). However, in embryos from flies expressing the Bsg25D coding region and 3' UTR (NGV; CR\textsubscript{3}'UTR), the chimeric mRNA localized to centrosomes in both the germline and the somatic region of the embryo (Fig. 3I). By contrast, eGFP- Bsg25D\textsuperscript{-CR} (NGV; CR) mRNA specifically associated with somatic centrosomes and was largely absent from pole cells (Fig. 3J), while eGFP- Bsg25D\textsuperscript{-3'UTR} (NGV; 3'UTR) mRNA was mostly enriched in pole cells (Fig. 3K). This suggests the presence of separable localization elements, one...
residing in the Bsg25D coding region mediating anterior/somatic targeting, and the other within the 3’ UTR of Bsg25D directing posterior localization, a pattern established during oogenesis.

**Mislocalization of Bsg25D affects microtubule polarity and Gurken deployment in the developing oocyte**

Niniein family members are involved in microtubule anchoring and nucleation. Therefore, we investigated whether mislocalizing Bsg25D would have an effect on microtubule arrangement in oogenesis, since microtubules are dynamic throughout oogenesis (Parton et al., 2011). We investigated microtubule organization in oogenesis since microtubules are dynamic throughout oogenesis. Therefore, we investigated whether mislocalizing Ninein family members are involved in microtubule anchoring and Dynein (MacDougall et al., 2003). In wild-type oogenesis Grk localizes to the antero-dorsal cortex at stage 8, forming a crescent around the oocyte nucleus (Neuman-Silberberg and Schüpbach, 1996). This localization pattern was observed in ovaries from Bsg25D

**Bsg25D can bind microtubules, and with Dynein can move along microtubules toward their minus-ends, in vitro**

Next, we explored the dynamics of Bsg25D association with microtubules in vitro. To determine whether Bsg25D can associate with microtubules, we performed in vitro experiments using purified microtubules and Bsg25D. The results indicated that Bsg25D can bind microtubules and move along them, indicating that it may play a role in regulating microtubule dynamics during oogenesis.
with microtubules, we used total internal reflection fluorescence microscopy (TIRF) to conduct live imaging of purified Bsg25D and microtubules (Gell et al., 2010). For these experiments, because we were unable to express full-length Bsg25D in bacteria despite repeated efforts, we used BsgN, the truncated form of Bsg25D containing only the N-terminal 353 amino acids (Fig. S4). We found that purified BsgN alone in BRB80 buffer bound efficiently to microtubules, unlike the control protein Dynein light chain 90f (Dlc90f), which requires Dynein intermediate chain (Dic) and Dynein heavy chain (Dhc) to bind to microtubules (Fig. 5A–D; Movies 1, 2) (Song et al., 2007). BsgN bound diffusely to microtubules, with no specific preference for minus- or plus-ends (Fig. 5C,D), and no binding events were ever observed for Dlc90f (Fig. 5A,B). Next, we used a microtubule pull-down assay (modified from Amrute-Nayak and Bullock, 2012; Lindesmith et al., 2001) to purify motor proteins from Drosophila embryo lysates (Fig. 5E). These isolated motor protein complexes were then imaged by TIRF microscopy to observe potential transport of Dlc90f and BsgN protein molecules. In this manner, we detected transport of both Dlc90f (Fig. 5F,G; Movie 3) and BsgN (Fig. 5H,I; Movie 4). Movement events were observed more frequently for BsgN than for the control protein Dlc90f (Fig. 5J; Movies 5, 6), and BsgN (Fig. 5K; Movies 7, 8).

Fig. 3. Bsg25D RNA contains localization elements within the coding region and 3′ UTR. (A) Schematic diagram of the transgenic constructs used, eGFP (green), Bsg25D-PB coding region (white), Bsg25D 3′ UTR (grey). (B–E) Distribution of transgenically-expressed mRNAs in stage 10 oocytes as shown by in situ hybridization, using a probe recognizing GFP, in the Bsg25D<sup>cr</sup>[Df(2L)6011 (−/Df) genetic background. The coding region alone promotes localization to the anterior pole, while the 3′ UTR promotes posterior localization. (F,G) Posterior localization is not apparent in early stage 10 oocytes expressing full-length GFP-Bsg25D (F) but is evident in similar stage oocytes expressing only GFP fused to the Bsg25D 3′ UTR (G). (H–K) Distribution of GFP-reporter mRNAs in early embryos expressing the transgenes as shown by in situ hybridization, using a probe recognizing GFP, in a wild-type genetic background. The 3′ UTR is essential for accumulation of these RNAs into the pole plasm and pole cells. All images scale bar=50 µm.
We measured the speed of Kin1 movement as 0.864±0.047 μm s\(^{-1}\) (means±s.e.m.; \(n=34\)), which is consistent with earlier measurements of its in vitro velocity (Howard et al., 1989). We also found that Dlc90f and Bsg\(^N\) moved at nearly identical speeds of 0.983±0.109 μm s\(^{-1}\) (\(n=23\)) and 0.978±0.074 μm s\(^{-1}\) (\(n=30\)), respectively, and these measurements are consistent with earlier analyses of Dynein movement in 1 mM ATP (Paschal et al., 1987; Ross et al., 2006).

Bsg25D functions in vivo to ensure accurate chromosome segregation during early embryonic nuclear divisions

Our analysis of hemizygous flies, in which the Bsg25D\(^{Null}\) allele was combined with a deficiency chromosome, Df(2L)Exel6011, which deletes the Bsg25D locus, revealed that Bsg25D\(^{Null}/Df(2L)Exel6011\) flies are viable and sufficiently fertile to be maintained as a stock. To evaluate more clearly whether Bsg25D loss-of-function impacts embryonic development, we next performed quantitative embryo viability assays following different crossing schemes. We first examined embryos from Bsg25D\(^{Null}/Df(2L)Exel6011\) females crossed to Bsg25D\(^{Null}/Df(2L)Exel6011\) males, which lack both maternally- and zygotically-expressed Bsg25D, and found however that approximately 70% failed to complete embryogenesis and did not hatch (Fig. 6A). We obtained similar results from embryos produced by Bsg25D\(^{Null}/Bsg25D\(^{Null}\) females crossed to
Bsg25DNull/Bsg25DNull males, and from Bsg25DNull/Bsg25DNull females crossed to Bsg25DN/Bsg25DN males. Complete viability was recovered when Bsg25DNull/Df(2L)Exel6011 females were crossed to wild-type males or when wild-type virgin females were crossed to Bsg25DNull/Df(2L)Exel6011 males (Fig. 6A). We conclude that Bsg25D function is required for embryonic development and that either the maternal contribution of Bsg25D, or its early zygotic expression, is sufficient for its function in embryogenesis.

We next examined the initial nuclear divisions in Bsg25DNull/Bsg25DNull embryos by staining their chromosomes with DAPI. This revealed that many such embryos exhibit excessive nuclear clearance from the embryo cortex (nuclear fallout) compared to wild-type controls (Fig. 6C,D). Moreover, Bsg25DNull/Bsg25DNull embryos showed abnormal nuclear aggregates, both large and small, and bridges between chromatids, indicating failed chromatid separation (Fig. 6B,E). These defects in nuclear division ranged from mild (Fig. 6B-E), where the normal uniform pattern of nuclear
divisions continues through the usual 13 rounds despite the phenotypes described above, to severe, where nuclear divisions fail resulting in embryonic lethality (Fig. 6B,G,H). Severely affected embryos do not cellularize and often display monopolar (not shown) and tripolar spindles (Fig. 6G,H). Many embryos were observed to have chromosome segregation defects in their initial nuclear cycles; however, in these early stage embryos the centrosomal marker γ-tubulin appeared to be localized normally, exhibiting a tight association with the mitotic spindle, as seen in wild-type specimens (Fig. 6F,G; Movie 9). In slightly older Bsg25DNull embryos, which had undergone more nuclear cycles, delocalization of the centrosome from the mitotic spindle can be
observed (Fig. 6H; Movie 10). This suggests that a subset of Bsg25D<sup>Null</sup> embryos fail to properly anchor centrosomes to the mitotic spindle, which may contribute to embryonic lethality.

Finally, to determine whether pole cell specification is particularly sensitive to Bsg25D function, we counted pole cells from embryos lacking Bsg25D that developed as far as the cellular blastoderm stage and from control embryos. We observed a modest decrease in pole cell number in progeny embryos from crosses among Bsg25D<sup>Null</sup>/Df(2L)Exc16011 male and female flies that was statistically significant with respect to wild-type controls (29±0.81 vs 34±1.97, P=0.010). When we made other similar comparisons, we also observed small decreases consequent to loss of Bsg25D activity that were however not statistically significant (Bsg25D<sup>Null</sup>/Df(2L)Exc16011; GFP/NGV, 29±1.87 pole cells, P=0.056 when compared with wild-type, and Bsg25D<sup>+/−</sup>/Bsg25D<sup>−/−</sup>, 30±1.58 pole cells, P=0.102 when compared with wild-type). We therefore cannot conclude that Bsg25D has a particular function in pole cell specification. However, since a significant number of embryos lacking Bsg25D activity are unable to complete more than nine rounds of nuclear divisions, and consequently do not reach the stage of development when pole cells would form, we could not include such embryos in our analyses.

**DISCUSSION**

In this work, we observed that a purified fragment of Bsg25D can bind to microtubules in vitro, and in the presence of purified motor proteins, move along them primarily in the minus-end direction at a velocity similar to that of Dynein. An association between Bsg25D and Dlc90f was previously identified in a high-throughput protein-protein interaction study (Giot et al., 2003). Our results are also consistent with results obtained with both Nin and Nlp, which bind to cytoplasmic Dynein through their N-termini (Casenghi et al., 2005). Targeting Nin and Nlp to the centrosome occurs through Dynein-mediated transport and is dependent on the microtubule cytoskeleton (Casenghi et al., 2005). While the overall direction of Bsg25D<sup>N</sup> movement was minus-end directed, we also documented instances of movement toward the plus-end of a microtubule. This is consistent with imaging data from live mammalian epithelial cells demonstrating bidirectional microtubule-directed movement for Nin (Moss et al., 2007). Bidirectional movement has been observed in other single molecule assays and is a well-established characteristic of Dynein (Amurte-Nayak and Bullock, 2012; Reck-Peterson et al., 2006). One potential difference between Bsg25D and its mammalian counterparts is that our in vitro experiments indicate that Bsg25D binds to microtubules in the absence of Dynein, which has not been demonstrated for Nin or Nlp. More targeted experiments could in the future establish the mechanistic relationship between Bsg25D and Dynein. These could include depleting for Dynein the motor protein complexes used for the TIRF imaging and determining whether there are effects on Bsg25D<sup>N</sup> mobility, or mapping and mutating the site on Bsg25D<sup>N</sup> necessary for Dynein binding, and then determining whether such a mutated protein can move along microtubules.

While Bsg25D has extensive sequence similarity to Nin and Nlp, it appears to be more closely related to Nin. Like both Nin and Nlp, Bsg25D contains numerous coiled-coil domains. In addition, one of three cAMP-dependent protein kinase (PKA) phosphorylation sites in Nin is conserved in Bsg25D (amino acids 124-130). This may be important for Bsg25D function, as Nin phosphorylation has been linked to centrosomal localization of certain Nin isoforms, and phosphorylation by PKA has been found to play a critical role in mitotic progression (Chen et al., 2003; Hong et al., 2000; Kotani et al., 1998; Lin et al., 2006). Bsg25D shares a conserved D-box domain with Nlp that is not present in Nin (Bsg25D amino acids 268-276), but it does not have the D-box or Ken-box motifs that for Nlp have been experimental shown to be important for cell cycle dependent degradation (Nlp amino acids 633-641 and 495-497) (Wang and Zhan, 2007). Nlp has phosphorylation sites for Aurora B or Cdc2/cyclin B1 kinases that do not appear to be conserved in Bsg25D, however independent mass spectrometry analyses of Nlp and Bsg25D reveals the presence of many phosphoserine and phosphothreonine residues at similar locations within both proteins (Casenghi et al., 2003; Zhai et al., 2008). Nlp phosphorylation by Plk is required for Nlp dissociation from the Dynein-Dynactin complex allowing for cell cycle progression in human cell lines (Casenghi et al., 2005).

While Bsg25D mutants can survive to become fertile adults, we observed that a majority of embryos that lack maternal and zygotic Bsg25D fail to hatch, and exhibit mitotic defects ranging from mild to very severe. Our experiments do not allow us to distinguish whether the mitotic defects are a cause or a consequence of the failure of many such embryos to develop. However, since Bsg25D associates with centrosomes, we can hypothesize that it contributes to their functions in microtubule nucleation and/or anchoring, and that its loss may cause abnormal mitotic spindle formation. While this manuscript was under review, we became aware of another study of Drosophila Bsg25D (Zheng et al., 2016). That paper reports similar results to ours with respect to localization of Bsg25D to centrosomes in early embryos. While both studies found Bsg25D null mutants to be viable, in contrast to our results their nin<sup>−/−</sup> allele did not produce a significant decrease in embryonic viability. This quantitative difference in our results could be a consequence of differences in culture conditions or genetic backgrounds.

A role for mammalian Nin in connecting microtubules to the centrosome has been proposed (Shinohara et al., 2013). As well, siRNA-mediated knockdown of Nin in human immortal cell lines resulted in mitotic catastrophe, cell cycle arrest in G2/M phase and apoptosis (Kimura et al., 2013). Given this severe phenotype, it is surprising that Bsg25D function is not required for viability in Drosophila under laboratory conditions, especially since only one Ninein-related protein is present in flies as opposed to two in mammals. In humans the rare disease Seckel syndrome-7 (SCKL7) is caused by missense mutations in the Nin gene (Dauber et al., 2012). SCKL7 results in a growth phenotype called microcephalic primordial dwarfism, which is a severe form of growth failure wherein growth restriction occurs in utero and continues after birth (Bober et al., 2010; Dauber et al., 2012). These patients, however, often survive until adulthood. Furthermore, Nlp has been linked to ciliopathies, Usher syndrome and Leber congenital amaurosis (van Wijk et al., 2009). Knockout mice for both Nin and Nlnl (which encodes Nlp) have been prepared (Brown and Moore, 2012) but they have not yet been studied in detail.

In Drosophila we also observed defects in Dhc and Grk localization upon overexpression and mis-localization of eGFP-Bsg25D mRNA in developing oocytes, which in turn led to an altered distribution of eGFP-Bsg25D protein. Polarization of microtubules within the developing oocyte is critical for transport of mRNAs necessary for axis determination in the early embryo. Our data suggest that overexpression and/or mislocalization of Bsg25D during oogenesis may affect microtubule-dependent localization processes, such as grk (MacDougall et al., 2003), and Dhc localization (Li et al., 1994), within the oocyte. Consistent with this, ubiquitous expression of Bsg25D with actin or tubulin Gal4 drivers results in early pupal lethality, also demonstrating that overexpression of Bsg25D is deleterious (Zheng et al., 2016).
Analogous results have been obtained for Nin and Nlp in mammalian cells. For example, in mammalian cultured cells overexpression of Nlp recruits γ-tubulin and hGCP4, a component of the γ-tubulin ring complex (γ-TURC) to ectopic loci, resulting in off-site microtubule nucleation and spindle formation (Casenghi et al., 2003). Overexpression of Nin has also been reported to lead to mis-localization of γ-tubulin in cultured human cells (Stillwell et al., 2004). Nlp overexpression is also frequently associated with cancer, including head and neck squamous cell carcinomas and ovarian cancer (Qu et al., 2008; Yu et al., 2009). In one study Nlp was found to be overexpressed in 80% of human breast and lung carcinomas that were investigated, and its overexpression led to tumorigenesis in transgenic mice (Shao et al., 2010).

In conclusion, our study of the dynamics of Bsg25D in vitro and of the consequences of its mutation or ectopic expression in an intact metazoan establish Drosophila as a model system for studying Ninein family proteins. Further work in this system will help reveal potential mechanisms through which loss or gain of function of Nin and Nlp might result in human disease.

MATERIALS AND METHODS

Drosophila strains

Oregon-R was used as wild-type for all experiments. The deficiency spanning Bsg25D (Df(2L)Exel6011, Blm7497) was received from the Bloomington Drosophila Stock Center. Truncated and null alleles of Bsg25D were generated using the ends-out gene targeting method, using the pw25.5 vector (generously provided by Dr David R. Hippler; Maggett et al., 2008). Primers used to produce Bsg25D FRT from D. melanogaster genomic DNA were: left arm Forward NotI-Bsg 5′-GGCGCGCCATCGAC-GGCGTATCGATAC-3′ and Acc61-Bsg 5′-GGATCCCTAACA-GAGGAGGGAGCCCT-3′; and right arm Asc1-Bsg 5′-GGCGCGCCGATTTGACGCT-3′ and Reverse Asc1-Bsg 5′-GG-GGGCGCCGGATAGAAGGTTGTGGTG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′. Primers for Bsg25D 5′N.sup were: left arm Forward Asc1-Bsg-Bsg 5′-GGTACCTGAGTACACTG-TAACGATCCATG-3′ and Reverse NotI-Bsg-Bsg 5′-GGCGCGCGGCCCATCG-3′. GAGGAGAGCCTCG-3′.
Dinkel, H., Van Roey, K., Michael, S., Kumar, M., Uyar, B., Altenberg, B., Milchevskaya, V., Schneider, M., Kühn, H., Behrendt, A. et al. (2016). ELM 2016–data update and new functionality of the eukaryotic linear motif resource. Nucleic Acids Res. 44, D294-D300.

Foe, V. E. and Alberts, B. M. (1983). Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in Drosophila embryogenesis. J. Cell Sci. 61, 31-70.

Fu, J. and Glover, D. M. (2012). Structured illumination of the interface between centriole and peri-centriolar material. Open Biol. 2, 120104.

Gell, C., Bormuth, V., Broughard, G. J., Cohen, D. N., Diaz, S., Friel, C. T., Helenius, J., Nitzsche, B., Petzold, H., Ribbe, J. et al. (2010). Microtubule dynamics reconstituted in vitro and imaged by single-molecule fluorescence microscopy. Methods Cell Biol. 95, 221-245.

Glot, L., Bader, J. S., Brouwer, C., Chaudhuri, A., Kuang, B., Li, Y., Hao, Y. L., Ooi, C. E., Godwin, B., Vitos, E. et al. (2003). A protein interaction map of Drosophila melanogaster. Science 302, 468-476.

Hadjidimou, K., Sonobe-Nojima, H., Tanigawa, A., Lasko, P. and Nakamura, A. (2008). Drosophila Pgc protein inhibits P-TEFb recruitment to chromatin in primordial germ cells. Nature 451, 730-733.

Hong, Y.-R., Chen, C.-H., Chou, M.-H., Liu, S.-Y. and Howung, S.-L. (2000). Genomic organization and molecular characterization of the human Ninein gene. Biochem. Biophys. Res. Comm. 279, 989-995.

Howard, J., Hudson, A. J. and Vale, R. D. (1989). Movement of microtubules by single kinesin molecules. Nature 342, 154-158.

Iampietro, C., Bergalet, J., Wang, X., Cody, N. A. L., Chinn, A., Lefebvre, F. A., Douziech, M., Krause, H. M. and Lécuyer, E. (2014). Developmentally regulated elimination of damaged nuclei involves a chk2-dependent mechanism of mRNA nuclear retention. Dev. Cell 30, 464-476.

Jones, J. and Macdonald, P. M. (2015). Neur14 contributes to germ cell formation and integrity in Drosophila. Biol. Open 4, 937-946.

Jongens, T. A., Hay, B., Jan, L. Y. and Jan, Y. N. (1992). The germ-cell-less gene product: a posteriorly localized component necessary for germ cell development in Drosophila. Cell 70, 569-584.

Kadyrova, L. Y., Habara, Y., Lee, T. H. and Wharton, R. P. (2007). Translational control of maternal Cyclin B mRNA by Nanos in the Drosophila germline. Development 134, 1519-1527.

Kimura, M., Yoshioka, T., Saio, M., Banno, Y., Nagaoaka, H. and Okano, Y. (2013). Mitotic catastrophe and cell death induced by depletion of centrosomal proteins. Cell Death Dis. 4, e603.

Kotani, S., Tugendreich, S., Fujii, M., Jorgensen, P.-M., Watanabe, N., Hoog, C., Hieter, P. and Todokoro, K. (1998). PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression. Mol. Cell 1, 371-380.

Lantz, V., Chang, J. S., Horabin, J. I., Bopp, D. and Schedl, P. (1994). The Drosophila orb RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. Genes Dev. 8, 598-613.

Lasko, P. (2012). mRNA localization and translational control in Drosophila oogenesis. Cold Spring Harb. Perspect. Biol. 4, a012294.

Lawo, S., Hasegawa, M., Gupta, G. D. and Pelletier, L. (2012). Subdiffraction imaging of centrosomes reveals higher-order organizational features of pericentriolar material. Nat. Cell Biol. 14, 1148-1158.

Letunic, I., Copley, R. R., Pils, B., Pinkert, S., Schultz, J. and Bork, P. (2006). SMART 5: domains in the context of genomes and networks. Nucleic Acids Res. 34, D257-D260.

Lécuyer, E., Yoshida, H., Parthasarathy, N., Alm, C., Babak, T., Cerovina, T., Hughes, T. R., Tomancak, P. and Krause, H. M. (2007). Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell 131, 174-187.

Lécuyer, E., Parthasarathy, N. and Krause, H. M. (2008). Fluorescent in situ hybridization protocols in Drosophila embryos and tissues. Methods Mol. Biol. 420, 289-302.

Lerit, D. A. and Gavis, E. R. (2011). Transport of germ plasm on astral microtubules directs germ cell development in Drosophila. Curr. Biol. 21, 439-448.

Lin, C.-C., Cheng, T.-S., Hsu, C.-M., Wu, C.-H., Chang, L.-S., Shen, Z.-S., Yeh, H.-M., Chang, L.-K., Howung, S.-L. and Hong, Y.-R. (2006). Characterization and functional aspects of human ninein isoforms that regulated by centrosomal targeting signals and evidence for docking sites to direct gamma-tubulin. Cell Cycle 5, 2517-2527.

Lindemuth, L. C., Kumar, J. and Sheeetz, M. P. (2001). Identification of kinesin-associated proteins. Methods Mol. Biol. 164, 205-212.

MacDougall, N., Clark, A., MacDougall, E. and Davis, L. (2003). Drosophila gurken (TGFr) mRNA localizes as particles that move within the oocyte in two centrosome-dependent steps. Dev. Cell 4, 307-319.
