**RESEARCH ARTICLE**

Kinesin-1 interacts with Bucky ball to form germ cells and is required to pattern the zebrafish body axis

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**ABSTRACT**

In animals, specification of the primordial germ cells (PGCs), the stem cells of the germ line, is required to transmit genetic information from one generation to the next. Bucky ball (Buc) is essential for germ plasm (GP) assembly in oocytes, and its overexpression results in excess PGCs in zebrafish embryos. However, the mechanistic basis for the excess PGCs in response to Buc overexpression, and whether endogenous Buc functions during embryogenesis, are unknown. Here, we show that endogenous Buc, like GP and overexpressed Buc-GFP, accumulates at embryonic cleavage furrows. Furthermore, we show that the maternally expressed zebrafish Kinesin-1 Kif5Ba is a binding partner of Buc and that maternal kif5Ba (Mkif5Ba) plays an essential role in germ line specification in vivo. Specifically, Mkif5Ba is required to recruit GP to cleavage furrows and thereby specifies PGCs. Moreover, Mkif5Ba is required to enrich Buc at cleavage furrows and for the ability of Buc to promote excess PGCs, providing mechanistic insight into how Buc functions to assemble embryonic GP. In addition, we show that Mkif5Ba is also essential for dorsoventral (DV) patterning. Specifically, Mkif5Ba promotes formation of the parallel vegetal microtubule array required to asymmetrically position dorsal determinants (DDs) towards the prospective dorsal side. Interestingly, whereas Syntabulin and wnt8a translational translation depend on kif5Ba, grip2a translation does not, providing evidence for two distinct mechanisms by which DDs might be asymmetrically distributed. These studies identify essential roles for maternal Kif5Ba in PGC specification and DV patterning, and provide mechanistic insight into Buc functions during early embryogenesis.

**KEY WORDS:** Bucky ball, Kinesin-1, Germ plasm, Dorsoventral patterning, Germ cell, Maternal

**INTRODUCTION**

In sexually reproducing animals, primordial germ cells (PGCs), germline stem cells that form the gametes, are specified early in development via induction by zygotic factors or inheritance of maternally deposited RNAs and proteins, termed germ plasm (GP) (Hartung and Marlow, 2014; Lesch and Page, 2012; Seervai and Wessel, 2013). Although key regulators of germline development are conserved, maternal inheritance modes utilize diverse regulators (e.g. oskar in insects and buc in vertebrates) and mechanisms of GP assembly (Hartung and Marlow, 2014; Lesch and Page, 2012; Seervai and Wessel, 2013). GP is both necessary and sufficient for germline specification (Hashimoto et al., 2004; Hathaway and Selman, 1961; Illmensee and Mahowald, 1974; Togashi et al., 1986), yet it remains mysterious how this process is regulated.

Maternal GP is produced in oocytes and assembled into ribonucleoprotein (RNP) complexes that are stabilized in specific subcellular locations (Hartung and Marlow, 2014). In zebrafish, maternal GP components, including *vasa*, *nanos3* and *dazl*, localize to the Balbiani body (Bb), an ancient asymmetric structure of primary oocytes (Howley and Ho, 2000; Kloc et al., 2004; Kosaka et al., 2007; Marlow, 2010). In zygotes, GP accumulates at distal cleavage furrows (Hashimoto et al., 2004; Knaut et al., 2000; Koprunner et al., 2001; Maegawa et al., 1999; Yoon et al., 1997), resulting in asymmetric inheritance of GP by four PGCs. After genome activation, GP is symmetrically inherited, resulting in four PGC clusters (Knaut et al., 2000). Although GP localization in zebrafish was first described nearly two decades ago, the molecular mechanisms governing GP inheritance remain elusive.

To date, zebrafish mutants disrupting GP assembly during early cleavages also perturb cleavages (Nair et al., 2013; Pelegri et al., 1999; Yabe et al., 2009). Both microtubule (MT) and F-actin networks are implicated in GP accumulation at cleavage furrows (Nair et al., 2013; Pelegri et al., 1999; Theusch et al., 2006; Yabe et al., 2009). MTs are enriched in distal cleavage furrows (Jesuthasan, 1998), and MT tips colocalize with GP-RNPs (Nair et al., 2013); however, it is unknown whether specific aggregation or stabilization factors exist and whether molecular motors are involved. Buc, a female germline-specific protein, is necessary and sufficient for Bb formation, in which GP localizes in primary oocytes (Bontems et al., 2009; Heim et al., 2014; Marlow and Mullins, 2008), and zebras and *Xenopus* Buc/Velo interact with RNAbhs that bind GP-RNAs (Heim et al., 2014; Nijjar and Woodland, 2013). Furthermore, when *buc*-gfp RNA is injected into embryos, the translated Buc-GFP protein localizes to cleavage furrows, like GP, and increases PGC numbers (Bontems et al., 2009), indicating that exogenous Buc can promote PGC formation. Although maternal *buc* mutants do exist (Dosch et al., 2004; Marlow and Mullins, 2008), the polarity defects of *buc* oocytes preclude loss-of-function (LOF) analysis of potential Buc roles in GP recruitment in embryos.

In teleosts and amphibians, dorsoventral (DV) patterning, like germline specification, depends on maternally provided RNAs and proteins called dorsal determinants (DDs) (Langdon and Mullins, 2011; Marlow, 2010) that reside within the Bb and undergo Buc-dependent Bb-mediated translocation to the vegetal cortex (Ge et al., 2014; Lu et al., 2011; Nojima et al., 2010). Following fertilization, MT reorganization underlies dorsal determination (Houlston and Elinson, 1991; Jesuthasan and Strähle, 1997), as vegetal parallel microtubule arrays (pMTAs) govern asymmetric redistribution of the vegetally localized DDs relative to the animal-vegetal axis (Ge et al., 2014; Gerhart et al., 1989; Houlston and...
kif5Ba recruits germ plasm RNAs to cleavage furrows and promotes germ cell specification

To test potential Kif5Ba involvement in localizing Buc, we generated zebrafish kif5Ba mutants using CRISPR-Cas9 mutagenesis (Chang et al., 2013; Hruscha et al., 2013) to disrupt the Kif5Ba motor domain (Fig. 1G). Two alleles were recovered and propagated. Sequencing of genomic DNA of F_1 progeny and cDNA of homozygous mutants identified a 5 base pair (bp) deletion predicted to produce a premature stop codon (kif5Ba<sup>ae11</sup>) and a 6 bp deletion predicted to delete two amino acids (kif5Ba<sup>ae12</sup>) (Fig. 1H). qRT-PCR revealed nonsense-mediated decay of kif5Ba<sup>ae11</sup> but not of kif5Ba<sup>ae12</sup> transcripts in homozygous mutants (Fig. 1I). Despite mild craniofacial defects of kif5Ba<sup>ae12</sup> transscripts in homozygous mutants (Fig. 1I), Kif5Ba<sup>ae11</sup> mutant larvae (data not shown), fertile adult homozygotes and transheterozygotes were recovered; thus, zygotic kif5Ba is dispensable through adulthood.

We next assessed germ cell specification in maternal kif5Ba mutants, hereafter called Mkif5Ba mutants, by examining GP-RNA localization in progeny of kif5Ba<sup>−/−</sup> females. During the first three cell cleavages, GP components accumulate at distal cleavage furrows and thereby become enriched in PGCs (Eno and Pelegri, 2013; Hashimoto et al., 2004; Knaut et al., 2000; Koprunner et al., 2001; Maegawa et al., 1999; Nair et al., 2013; Yabe et al., 2009; Yoon et al., 1997). The GP-RNAs nano3 and vasa accumulated at the cleavage furrows of 4-cell-stage embryos (Fig. 2A,E) and later in PGCs of shield-stage WT (Fig. 2C,G), but not at the furrows of Mki5Ba embryos (Fig. 2B,F); and thus, no PGCs were detected in Mki5Ba mutants (Fig. 2D,H). Conversely, vegetal localization of dash RNA was intact in Mki5Ba mutant embryos (Fig. 2J), suggesting that vegetal localization occurs independently of kif5Ba. Although GP-RNAs were absent from furrows, RT-PCR confirmed their presence in WT and Mki5Ba embryos through 2 h post-fertilization (hpf) (Fig. 2K), suggesting that kif5Ba mediates furrow enrichment.

PGCs reach gonad anlagen at 30 hpf (Raz and Reichman-Fried, 2006). In WT embryos, Vasa<sup>+</sup> PGCs localized adjacent to the yolk extension (Fig. 2L,M), but were absent in Mkif5Ba mutants (Fig. 2N,O), indicating failed PGC specification. Conversely, Mkif5Ba embryos developed exclusively as sterile males (Table 1) devoid of germ cells (Fig. 2P-Q<sup>−</sup>). These results indicate that maternal kif5Ba is essential for GP recruitment to cleavage furrows, for PGC specification and for fertility.

Germ plasm RNA localization is intact in kif5Ba mutant oocytes

In primary oocytes, GP components are enriched in the Bb (Hartung and Marlow, 2014; Kosaka et al., 2007) in a Bb-dependent manner (Bontems et al., 2009; Heim et al., 2014; Marlow and Mullins, 2008). After Bb translocation to the vegetal pole, it disperses in stage-II oocytes; thereafter, GP component localization diverges (Hartung and Marlow, 2014; Braat et al., 1999; Knaut et al., 2000; Kosaka et al., 2007; Draper et al., 2007; Howley and Ho, 2000; Maegawa et al., 1999). RT-PCR analysis indicated that GP-RNAs were present in embryos; however, impaired GP-RNA localization in oocytes could cause GP defects in embryos. Buc protein localization and H&E staining, labeling with the ER and mitochondria marker DiOC6, confirmed that oocyte polarity was intact in kif5Ba<sup>−/−</sup> primary oocytes (supplementary material Fig. S1A,B,E-H). Later-stage oocytes also appeared grossly normal (supplementary material Fig. S1C,D). Fluorescent in situ hybridization (FISH) (Gross-Thebing et al., 2014) revealed enrichment of vasa and nano3 RNAs in the Bbs of WT and kif5Ba<sup>−/−</sup> mutants (supplementary material Fig. S1E,F).
Fig. 1. Endogenous Buc localizes to the germ plasm of embryos and binds Kinesin-1. (A,B) Endogenous Buc localizes to distal cleavage furrows of 4-cell embryos when chromosomes are decondensed (A), but not during metaphase (B) or anaphase. Inset shows DAPI. Arrowheads indicate Buc accumulation. Scale bars: 100 µm for main images, 10 µm for insets. See text for quantification. (C) GFP-Buc, but not GFP, co-immunoprecipitates HA-Kif5Ba in HEK293T cells. Error bars show mean±s.e.m.; Student’s t-test, *P=0.0388. (D-F) Overexpressed GFP-Buc localizes to cleavage furrows and overexpressed HA-Kif5Ba is expressed throughout blastomeres, potentially allowing them to interact in vivo. Arrowheads indicate Buc accumulation. (G) Schematic protein structure of Kif5Ba, illustrating motor, stalk and tail domains. Red dashed line indicates CRISPR target site. (H) DNA and predicted protein sequences of WT and mutant kif5Ba alleles (ae11 and ae12). Yellow indicates protospacer, orange indicates PAM, gray area/red dashed line indicates a deletion and pink indicates altered amino acids. (I) qRT-PCR for kif5Ba RNA from pools of 5-dpf WT and homozygous mutants. kif5Ba undergoes nonsense-mediated decay in kif5Ba ae11/ae11 but not kif5Ba ae12/ae12 mutants. Error bars show mean±s.e.m.; Student’s t-test, *P=0.0388.

material Fig. S1I-L,O-R). Moreover, vasa and nanos3 were comparable in WT and kif5Ba ae11/ae11 oocytes after Bb dispersal (supplementary material Fig. S1M,N,S,T). Together, these results show that kif5Ba is dispensable for animal-vegetal polarity and GP-RNA localization in oocytes, suggesting that maternal kif5Ba probably acts to recruit GP in embryos.

Cleavage furrows appear normal in M kif5Ba mutants

Several lines of evidence indicate that cleavage furrow integrity and cytoskeletal architecture are crucial for GP recruitment to furrows. First, maternal-effect mutants that disrupt cytokinesis disrupt GP aggregation (Nair et al., 2013; Pelegri et al., 1999; Yabe et al., 2009). Second, during cytokinesis the cleavage furrow is flanked by MTs oriented perpendicular to the furrow, known as the furrow microtubule array (FMA), (Jesuthasan, 1998). During furrow maturation, FMA enrichment resembles that of GP, and angled MTs distally (supplementary material Fig. S2G,H). These results indicated that failed GP recruitment in M kif5Ba embryos was not due to furrow-associated cytoskeleton abnormalities.

Providing exogenous kif5Ba to M kif5Ba mutants following fertilization rescues PGC specification

Because oocytes and cleavage furrows were intact, and GP components were available but not recruited in M kif5Ba embryos, we hypothesized that injection of kif5Ba RNA into M kif5Ba embryos might rescue GP recruitment and PGC specification. Unlike WT embryos, which recruited nanos3 RNA to the first three cleavage furrows (Fig. 3A; n=22/24 embryos), enriched nanos3 RNA in PGCs at sphere stage (Fig. 3D; 3.14±0.41 PGCs/embryo; n=28 embryos) and had clusters of Vasa+ PGCs at 30 hpf (Fig. 3G; 13.24±4.09 PGCs/side; n=21 embryos), M kif5Ba mutants failed to recruit nanos3 to cleavage furrows (Fig. 3B; n=18/18 embryos) and thus lacked PGCs at sphere stage (Fig. 3E; 0.01±0.01 PGCs/embryo; one PGC in n=1/73, zero in n=72/73 embryos) and 30 hpf (Fig. 3H; zero PGCs in n=35/35 embryos). Injection of ha-kif5Ba RNA into M kif5Ba embryos, however,
promoted nanos3 RNA recruitment to one or two furrows (Fig. 3C; n=6/32 embryos), and a small number of PGCs were detected at sphere stage (Fig. 3F; 0.15±0.04 PGCs/embryo; one PGC in n=13/85, zero in n=72/85 embryos) and 30 hpf (Fig. 3I; n=6/45 embryos with PGCs; 2.00±1.10 PGCs/side in those embryos with PGCs). Importantly, injection of RNA encoding Cherry had no effect on PGC number in WT nor did it rescue PGC number in Mkie5Ba mutants (supplementary material Fig. S3). Together, these results suggest that Kif5Ba functions after fertilization to mediate GP recruitment to the cleavage furrows.

**Kif5Ba localizes Buc to cleavage furrows and mediates germ plasm assembly in embryos**

Because kif5Ba RNA injection restored GP recruitment in Mkie5Ba mutants, we postulated that Kif5Ba localizes a GP recruitment or aggregation factor. Based on its localization to cleavage furrows...
(Fig. 1), its ability to promote excess PGCs when overexpressed (Bontems et al., 2009), and its interaction and colocalization with Kif5Ba (Fig. 1), we hypothesized that Kif5Ba might mediate GP recruitment or stabilization by localizing Buc. Therefore, we injected GFP-Buc into 1-cell-stage WT or M{kif5Ba} mutant embryos and examined its localization. GFP-Buc accumulated at distal cleavage furrows of WT embryos (n=32/32), as previously reported (Bontems et al., 2009), but was not recruited in most M{kif5Ba} mutants (Fig. 4A,B; n=45/62). When present at the furrows of mutants, GFP-Buc did not aggregate distally but remained distributed along the furrow (n=17/62). Like GFP-Buc, endogenous Buc was enriched at distal furrows of WT (n=24/32 have Buc at ≥1 furrow), but this rarely occurred in M{kif5Ba} mutants (n=4/32 have Buc at ≥1 furrow) (Fig. 4C,D). Together with our binding data, these results suggest that Kif5Ba enriches Buc and GP at cleavage furrows.

Next, we tested whether Kif5Ba was required to increase PGC numbers in embryos overexpressing Buc. As previously reported (Bontems et al., 2009), additional nanos3+ cells were detected in GFP-Buc-injected WT compared with uninjected siblings at 4 hpf (Fig. 4E,F; n=10 uninjected and injected embryos with averages of 4.0 and 5.7 cells, respectively; P=0.0314, Student’s t-test).

By contrast, GFP-Buc produced no increase in nanos3+ cells in M{kif5Ba} mutants (Fig. 4G,H; n=3/63 uninjected and 6/50 injected: averages of 0.05 and 0.14 PGCs, respectively; P=0.1221, Student’s t-test). These results indicate that Kif5Ba is required to promote excess PGC formation in response to Buc overexpression and that the presence of Buc at the cleavage furrows is integral to GP assembly.

### Maternal kif5Ba is required for proper dorsoventral patterning

In addition to lacking germ cells, M{kif5Ba} mutants were ventralized to varying degrees at 1 day post-fertilization (dpf) (Fig. 5A-F), ranging from lack of notochord (V1, Fig. 5B) to complete axis radialization (V5, Fig. 5F). Additionally, a subset had secondary axes with duplicated rostral structures and a fused caudal body (Fig. 5G,5’), reminiscent of M{grip2a} mutants (Ge et al., 2014). Because ventralization was only observed in progeny of homozygous kif5Baae11/ae11 and kif5Baae12/ae12 and transheterozygous kif5Baae11/ae12 females, but not WT or heterozygous genotypes, we concluded that kif5Ba mutation caused ventralization (Fig. 5H).

### Table 1. M{kif5Ba} and MZ{kif5Ba} adult mutants are sterile males

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<th>Genotype</th>
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<td>100% (n=8)</td>
<td>100% unfertilized (N=4 adults screened, n=360 eggs total)</td>
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<td>kif5Ba ae11/ae12</td>
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and wnt8a RNA were vegetally localized at 2 min post-egg activation (mpa) (Fig. 6A,F), and by 45 mpa grip2a and wnt8a RNA, but not sybu RNA, asymmetrically translocated in WT (Fig. 6C,H,L). In M{kif5Ba} mutants, sybu RNA was initially vegetally localized (Fig. 6B), but was more dispersed [sybu domain angle in M{kif5Ba} (n=57 eggs; 3 females) was 1.14-fold that of WT (n=57 eggs; 4 females); Student’s t-test: P=6.78×10^-30]. At 45 mpa, two sybu RNA expression classes, strong and weak, were observed in both WT and M{kif5Ba} mutants, although more M{kif5Ba} mutants showed weak expression (Fig. 6D-C). qRT-PCR for sybu RNA confirmed that it was significantly elevated in M{kif5Ba} mutants at 2 mpa, and, although not significantly different, trended towards lower levels at 45 mpa (Fig. 6E). As no transcription occurs during this period, these results indicate that sybu RNA is not properly maintained in M{kif5Ba} mutants. Unlike sybu RNA, in M{kif5Ba} activated eggs grip2a and wnt8a RNA localization resembled WT initially (Fig. 6G,K; grip2a RNA mutant angle was 0.98±0.03 times that of WT and wnt8a RNA mutant angle was 1.07±0.05 times that of WT; Student’s t-test, P=0.77 and P=0.43, respectively); however, by 45 mpa, grip2a translocated asymmetrically in M{kif5Ba} activated eggs (Fig. 6L,N), but wnt8a did not (Fig. 6M,N). These data suggest that kif5Ba maintains sybu RNA at the vegetal pole and is necessary for wnt8a, but not grip2a, RNA translocation.

Fig. 4. Kif5Ba localizes Buc to cleavage furrows to mediate germ plasm assembly in the embryo. (A,B) GFP-Buc is recruited to distal furrows of WT (arrowheads in A) but not of M{kif5Ba} mutants (B). [n=0/29 for M{kif5Ba} (N=2 females), n=0/18 for M{kif5Ba} kif5Ba^ae11/ae12 (N=3 females), n=0/15 for kif5Ba^ae12/ae12 (N=1 female)], n=32/32 for WT (N=4 females) have compact distal GFP-Buc; n=2/29 for M{kif5Ba} kif5Ba^ae11/ae12, n=15/18 for M{kif5Ba} kif5Ba^ae11/ae12, n=0/15 for kif5Ba^ae12/ae12 have some furrow recruitment of GFP-Buc, although distal furrow compaction is defective.] (C,D) Endogenous Buc localizes to distal cleavage furrows of WT (arrowheads in C) but not M{kif5Ba} mutants (D). [n=2/15 for M{kif5Ba} kif5Ba^ae11/ae12 (N=2 females), n=2/17 for M{kif5Ba} kif5Ba^ae11/ae12 (N=1 female), n=24/32 for WT (N=4 females) of cells with decondensed chromosomes have Buc at ≥1 distal cleavage furrow.] (E-H) Compared with un.injected WT (E), gfp-buc injected WT (F) embryos have additional nanos^3^ PGCs (arrowheads), whereas uninjectected (G) and gfp-buc-injected (H) M{kif5Ba} mutants lacked nanos^3^ cells. See text for quantification.

Heterozygotes for either allele. Together, these results are consistent with both kif5Ba^ae11 and kif5Ba^ae12 having LOF character, with kif5Ba^ae12 probably being a stronger LOF allele than kif5Ba^ae11. Although unlikely, we cannot exclude the possibility that kif5Ba^ae12 acts as a weak dominant negative allele by dimerizing with other Kifs to form non-functional motors or competing for cargo.

**kif5Ba localizes dorsal determinants in the activated egg**

Dorsal specification in zebrafish is apparent around the mid-blastula transition and can be detected by nuclear kif5Ba localization Syntabulin (Sybu) protein translocates after egg activation (Fig. 6O,P) (Nojima et al., 2010). In M{kif5Ba} activated eggs, Syb protein, like sybu RNA, was mislocalized in some M{kif5Ba} eggs at 2 mpa, either aberrantly present on the lateral cortex or diffuse about the cortex (Fig. 6Q-Q'); Sybu was vegetally localized in n=3/12 M{kif5Ba} kif5Ba^ae11/ae12, n=4/9 M{kif5Ba} kif5Ba^ae11/ae11 and n=2/9 M{kif5Ba} kif5Ba^ae12/ae12 compared with n=13/15 WT eggs). At 45 mpa, in a subset of eggs, Sybu either failed to translocate or was not detectable (Fig. 6R-R'); Sybu translocated normally in n=1/11 M{kif5Ba} kif5Ba^ae11/ae12, n=12/18 M{kif5Ba} kif5Ba^ae11/ae11 and n=3/11 for M{kif5Ba} kif5Ba^ae12/ae12 compared with n=16/19 WT eggs). Thus, kif5Ba might function to maintain vegetal localization of Syb and for its subsequent asymmetric movement. Because Syb can link Kif5s and their cargo in other biological contexts (Cai et al., 2005; Su et al., 2004), and zebrafish Syb and Kif5B interact in vitro (Nojima et al., 2010), Syb probably localizes in oocytes or early embryos via its interaction with Kif5Ba.

To determine when Kif5Ba acts in DV patterning, we attempted to rescue the M{kif5Ba} ventralization phenotype by injecting ha-kif5Ba RNA into M{kif5Ba} embryos; however, this did not suppress the penetrance or expressivity of M{kif5Ba} ventralized phenotypes (Fig. 6S). Notably, other maternal-effect mutants that disrupt early translocation events also cannot be rescued by RNA injection after fertilization (Ge et al., 2014; Nojima et al., 2010). Together, these results are consistent with roles for Kif5Ba in positioning Syb in oocytes or during egg activation.

**Vegetal microtubules fail to organize in kif5Ba mutants**

Prior to asymmetric translocation of Syb protein and wnt8a RNA (~20 mpa), the vegetal MT network reorganizes to form the vegetal pMTA (Jesuthasan and Strähle, 1997), which predicts the DV axis (Tran et al., 2012). Disrupting this network, as occurs in M{grip2a} mutants (Ge et al., 2014), or by treating activated eggs with MT inhibitors, cold temperature or UV irradiation (Lu et al., 2011; Nojima et al., 2010), blocks Syb protein and wnt8a RNA translocation. To determine whether M{kif5Ba} DV patterning defects were due to pMTA defects we analyzed the MT network of
activated eggs. Surprisingly, although pMTA formation was robust in WT (Fig. 7A,M), it failed in most Mkif5Ba activated eggs (Fig. 7D, M). Instead, Mkif5Ba vegetal MTs either appeared bundled and randomly oriented (Fig. 7G,M) or non-bundled with a crosshatch appearance (Fig. 7I,M). No differences were observed between the lateral cortex MT networks of WT and Mkif5Ba eggs (Fig. 7N,Q).

Cortical granules (CGs) are normally rapidly exocytosed following egg activation (Becker and Hart, 1999). Persisting CGs in zebrafish maternal-effect mutants disrupting heterogeneous nuclear ribonucleoprotein I (hnRNPI) are thought to interfere with vegetal pMTA formation and disrupt DV patterning (Mei et al., 2009). To determine whether persisting CGs could explain the disrupted pMTA in Mkif5Ba mutants, CGs were labeled with *Maclura pomifera agglutinin* (MPA) lectin (Becker and Hart, 1996, 1999). Mkif5Ba activated eggs at 25 mpa displayed variable CG exocytosis impairment (Fig. 7E,H,K); however, quantification of the number of retained CGs within a defined vegetal region revealed no difference in the density of CGs retained between Mkif5Ba mutants, regardless of pMTA defect severity (Fig. 7M). Specifically, Mkif5Ba mutants with severe CG exocytosis deficits but normal vegetal pMTAs and Mkif5Ba mutants with mild CG exocytosis defects but disorganized MTs were observed (Fig. 7M). Furthermore, consistent with their more severe ventralization phenotypes, pMTA defects were more severe in Mkif5Ba<sup>ae12/aec12</sup> mutants than Mkif5Ba<sup>ae11/aec11</sup> mutants; however, the number of retained CGs was not significantly different between these genotypes (supplementary material Fig. S4A,B). Together, these data indicate that maternal *kif5Ba* mediates pMTA formation and CG exocytosis, but persisting CGs do not account for the cytoskeletal defects.

**DISCUSSION**

Our study identifies the key maternal motor required for specification of the germline and the DV axis in zebrafish (Fig. 8). Moreover, our findings that Kif5Ba binds to Buc, mediates Buc recruitment to the cleavage furrow, where GP assembles in the embryo, and is required for excess PGC formation in response to Buc overexpression, provide new insight into the mechanism by which Buc promotes germ plasm assembly in embryos. Furthermore, our studies show that Kif5Ba-mediated MT reorganization underlies cortical rotation-like events to promote asymmetric localization of Syntabulin and wnt8a, and that asymmetry of *grip2a* occurs independently of *kif5Ba*. Thus, our results provide evidence for distinct MT-dependent translocation events that mediate maternal DV patterning.

**A Kif5Ba-Buc interaction required for germ plasm assembly and germline specification**

Although germline development in all animals relies on conserved genetic factors, two main modes of germline specification exist (Ewen-Campen et al., 2010; Lesch and Page, 2012; Seervai and Wessel, 2013). Germline stem cells are either induced by zygotic factors or rely on the inheritance of maternal GP to specify PGCs (Dosch, 2014; Hartung and Marlow, 2014; Marlow, 2010). In zebrafish, GP-RNAs and proteins localize to the Bb of primary oocytes in a Buc-dependent manner (Bontems et al., 2009; Heim et al., 2014; Kosaka et al., 2007; Marlow and Mullins, 2008) and are later inherited and stabilized in the distal cleavage furrows during the first embryonic cleavage cycles (Hashimoto et al., 2004; Knaut et al., 2000; Koprüner et al., 2001; Maegawa et al., 1999; Theusch et al., 2006; Yoon et al., 1997). Manual ablation of GP prevents PGC specification (Hashimoto et al., 2004), indicating that it is essential for fertility; however, the mechanisms governing embryonic GP segregation are poorly understood.

Along with the absence of overt defects in oocyte polarity and GP localization in *kif5Ba* mutant ovaries, our *ha-kif5Ba* RNA injection rescue experiments provide compelling evidence that Kif5Ba functions during cleavage stages to localize GP components. However, RNA injection only partially suppresses deficits. Because
GP recruitment to the furrows only occurs between the 2-cell stage and 16-cell stage, the rescue activity of ha-kif5Ba RNA is probably limited by the time required to produce sufficient functional Kif5Ba protein during this 1.5-h window. Nonetheless, it is clear that exogenous kif5Ba RNA provided following fertilization can promote GP recruitment to cleavage furrows of 16-cell embryos, suggesting that endogenous Kif5Ba performs this function during the first three cleavages. Significantly, this is the first maternal factor identified in zebrafish that is required for GP recruitment which does not interfere with the furrow-associated cytoskeleton or cleavage furrow formation.

We have shown that endogenous Buc, a necessary factor for GP assembly in oocytes, localizes, like GP, to distal cleavage furrows of embryos. Furthermore, loss of Buc localization to furrows, as occurs in Mkif5Ba mutants, coincides with failed GP recruitment and ultimately failure to specify PGCs. Additionally, although Buc overexpression can promote PGC formation in WT (Bontems et al., 2009), we show that this activity requires MKif5Ba; therefore, Buc probably acts at the furrow to recruit or stabilize GP. Because Buc interacts with RNA-bps (Heim et al., 2014), it might recruit GP-RNPs indirectly, although we cannot exclude the possibility that Buc possesses uncharacterized RNA binding elements.
Our observation that Buc localization to distal furrows is dynamic, occurring preferentially when chromosomes are decondensed, is consistent with a model whereby Buc seeds GP recruitment and assembly until sufficient GP proteins and RNAs accumulate and Buc is no longer required. Additionally, the distal cleavage furrows are enriched with MTs that form V-shaped structures oriented toward the site of GP assembly during furrow maturation (Jesuthasan, 1998; Urven et al., 2006). Because we observed interactions between Kif5Ba and Buc, it is tempting to speculate that Kif5Ba might mediate transport of Buc to the furrows along these MTs. It is possible that cytoskeletal reorganization accompanying furrow maturation could subsequently account for the dynamic localization of Buc.

Maternal Kif5Ba mediates vegetal microtubule reorganization and the cortical rotation-like event

In zebrafish and Xenopus, DV axis specification requires cytoskeletal reorganization to form a pMTA that facilitates asymmetric localization of maternal DDs (Houliston and Elinson, 1991; Jesuthasan and Strähle, 1997). In zebrafish, previous work shows that maternal grip2a, sybu and wnt8a regulate DV patterning (Ge et al., 2014; Lu et al., 2011; Nojima et al., 2010). Grip2a is implicated in pMTA formation, and Syntubulin is hypothesized to tether vegetally localized DDs, such as wnt8a (Ge et al., 2014; Lu et al., 2011; Nojima et al., 2010). However, little is known about how these events are regulated.

Our observation that vegetal pMTA formation fails in Mkif5Ba mutants probably accounts for deficits in wnt8a localization and, ultimately, ventralization of mutant progeny. Interestingly, asymmetric localization of grip2a is intact in Mkif5Ba mutants. Previous studies indicate that grip2a translocation is disrupted by nocodazole, which disrupts formation of the vegetal pMTA (Ge et al., 2014). Thus, it appears that distinct MT-dependent mechanisms asymmetrically localize cargoes in activated eggs: a Kif5Ba-independent mechanism used by grip2a, and a Kif5Ba- and pMTA-dependent mechanism used by wnt8a and Sybu. These results raise the interesting possibility that movement of grip2a RNA might anticipate vegetal pMTA formation. Interestingly, Grip1, a grip2a family member, directly interacts with Kif5s (Setou et al., 2002). Therefore, following grip2a RNA translocation, an asymmetric Kif5Ba-Grip2a complex might mediate pMTA formation. Alternatively, Kif5Ba could mediate pMTA formation and bundling by crosslinking MTs using its head and tail MT-binding elements. Such a configuration could also support the zebrafish cortical rotation-like event by MT-MT sliding, as has been described for Kinesin-1 in other contexts (del Castillo et al., 2015; Jolly et al., 2010).

Our finding that kif5Ba is required to maintain Sybu at the vegetal cortex and for its subsequent asymmetric translocation supports previous work showing that Sybu interacts with Kif5Ba (Nojima et al., 2010). We also observed that sybu RNA was present at higher levels and appeared more dispersed in Mkif5Ba mutants following...
Fig. 8. Model for Kif5Ba functions in dorsoventral patterning and germ cell formation. In WT activated eggs Kif5Ba mediates vegetal pMTA formation and dorsal determinant (DD) translocation towards the prospective dorsal side. Kif5Ba localizes endogenous Buc to distal cleavage furrows and promotes recruitment of GP-RNAs to furrows and subsequent PGC specification. In Mkif5Ba mutants the vegetal pMTA fails to form, resulting in ventralization. Furthermore, Buc localization to furrows is lost, resulting in failed GP recruitment to specify PGCs and adult sterility. Our data support a model whereby Kif5Ba is required to recruit Buc and GP-RNAs to cleavage furrows, although it is unclear whether it also acts to compact GP-RNAs within distal regions of the cleavage furrow.

egg activation, and that these increased levels were not maintained. Although it is unclear why sybu RNA would initially be more abundant, a role for MKif5Ba in tethering sybu RNA at the vegetal cortex to protect it from degradation might explain its dispersal and lack of maintenance. Notably, because Sybu was mislocalized at 2 mpa but vegetal localization of grip2a and wnt8a were intact, this suggests that Sybu probably does not tether these RNAs. It remains possible that Sybu tethers Grip2a or Wnt8a proteins or other uncharacterized DDs. Alternatively, Sybu might contribute to DV patterning by another mechanism.

MATERIALS AND METHODS

Animals

AB strain wild-type zebrafish embryos were obtained from natural pairwise matings and were reared according to standard procedures (Westerfield, 2000). Embryos were raised in 1× Embryo Medium at 28.5°C and staged as described (Kimmel et al., 1995). All procedures and experimental protocols were in accordance with NIH guidelines and approved by the Einstein IACUC (protocol #20140502).

Mutagenesis

kif5Ba zebrafish mutants were created using CRISPR-Cas9-mediated mutagenesis (http://www.addgene.org/crispr/jounglab/CRISPRzebrafish/) (Hwang et al., 2013a,b). A guide RNA (gRNA) targeting the motor domain of kif5Ba was designed using ZifIT Targeter software (http://zifit.partners.org/ZifIT/). The primers gRNA-F: TTAGGACAGGATAGCGTC- GTGAT and gRNA-R: AAAACATCGACGCTATCCGTG were annealed and ligated into Bsal-HF-linearized pDR272 to create pDR272-kif5Ba-gRNA. Oligo insertions were sequence-verified, and gRNAs were synthesized using MAXIscript T7 Kit (Life Technologies, AM1312M) on DraI-linearized pDR272-kif5Ba-gRNA. Cas9 RNA was synthesized using mMESSAGE mMACHINE SP6 Transcription Kit (Life Technologies, AM1340) on MorI-linearized MLM3613 and poly-Adenylated with the Poly(A) Tailing Kit (Life Technologies, AM1350). gRNA and Cas9 RNA were co-injected (2 nl at 12.5 ng/μl and 300 ng/μl, respectively). T7 endonuclease I assays (Hwang et al., 2013b) and sequencing were used to determine mutagenesis efficiency. Injected embryos were raised to adulthood, their progeny were screened, and two founders bearing distinct mutations were identified and propagated.

Genotyping

Genomic DNA was extracted from adult fins or embryos using standard procedures (Westerfield, 2000). The region surrounding the kif5Ba<sup>wt</sup> and kif5Ba<sup>ae11</sup> and kif5Ba<sup>ae12</sup> mutations was amplified with primers 5’-GGAGTGCACCATT-AAAAGTCATGTG and 5’-GTCGGTTGCAAATATTGAGGTC. Restriction enzyme (RE) digestion with PvuI for kif5Ba<sup>ae11</sup> produced two smaller fragments for the mutant allele. RE digestion with Mbol for kif5Ba<sup>ae12</sup> produced two smaller fragments for the wild-type allele but not the mutant allele.

In situ hybridization

For chromogenic in situ hybridization, embryos or activated eggs at the specified stages were fixed in 4% paraformaldehyde overnight at 4°C. In situ hybridization was performed according to Thise and Thise (2014), except that maleic acid buffer (100 mM maleic acid, pH 8, 150 mM NaCl) was substituted for PBS during antibody incubations and BM Purple was used to develop (Roche, 1442074). FISHs were performed using the Multiplex Fluorescent Reagent Kit (ACD Bio, 320850) according to the whole-mount RNAscope protocol (Gross-Thebing et al., 2014). Dissected ovaries were fixed for 30 min in 4% paraformaldehyde at room temperature (RT), washed with PBT (0.1% Tween), dehydrated in MeOH and placed at −20°C.
Eppendorf realplex2 Mastercycler. Expression relative to WT was ATGCCATGATTGTCTCCACA. qRT-PCR reactions were performed in GATGGAAGA; (Neumann et al., 2011) and lysed in RIPA buffer. To pre-clear the lysate and beads were then washed with RIPA. Proteins were eluted in 60 μl SDS-BME; y1165 (Heim et al., 2014) were simultaneously incubated at 4°C for 1 h. Pre-cleared lysate and antibody-bound beads were incubated at 4°C for 1 h and beads were then washed with RIPA. Proteins were eluted in 60 μl SDS-BME at 70°C for 5 min. Eluates were run on pre-cast polyacrylamide gels (Bio-Rad, 161-1174), stained with Gel Code Blue stain (Pierce, PI24592), and bands were extracted and analyzed with mass spectrometry and Scaffold software (Einstein Proteomics Facility).

**Co-immunoprecipitation in HEK293T cells**

HEK293T cells were transfected with pCS-GBP-Buc and pCS2+HA-Kif5Ba (Nojima et al., 2010) using OptiMem (Life Technologies, 31985) and PEI, and lysed at 48 h post-transfection using RIPA plus protease-inhibitors (Sigma, P2714). Lysates were pre-cleared with Protein-G Beads (New England Biolabs, S1430S) at 4°C for 1 h, then immunoprecipitated with anti-GFP antibody clone 3E6 (Life Technologies, A11120). Eluates were run on Fisher EZ-Run 12.5% SDS-PAGE (Fisher, BP7712) and blotted with anti-HA 3F10 antibody (Roche, 1186743001). Proteins were detected with HRP-conjugated anti-Rat IgG (H+L) (Jackson ImmunoResearch, 112-036-003), using the Amersham ECL Plus Chemiluminescence kit (GE Healthcare, RP2132).

**Image acquisition and processing**

Whole-mount chromogenic in situ hybridization and live whole-mount 1 dpf embryos were imaged using an Olympus SZ61 dissecting microscope with a high-resolution digital camera (model S97809, Olympus America) and Picture Frame 3.0 software (Optronics). H&E stainings were imaged using a Zeiss Axioscope microscope with a Zeiss AxioCam MRc camera and Zeiss AxioVision Rel. 4.6 software. Anti-Syntubulin-stained eggs, RNAscope in situ hybridization, cytokeratin at the cleavage furrows, oocyte immunofluorescence and anti-Vasa-stained embryos were imaged using a Zeiss 5Live Duoscan line-scanning confocal microscope.

**Acknowledgements**

We thank F. Pelegri, M. Hibi and H. Knaut for reagents, Marlow lab members for discussions, our animal staff, and the Analytical Imaging (NIC P30CA13330), Histotechnology and Proteomics Facilities for technical support.

**Competing interests**

The authors declare no competing or financial interests.

**Authors contributions**

P.D.C. and M.Z.S. identified kif5Ba alleles. P.D.C., A.E.H. and F.L.M. performed dissections and histological analysis. P.D.C. and A.E.H. performed all other experiments and analysis in consultation with F.L.M. P.D.C., A.E.H. and F.L.M. conceived and designed experiments. F.L.M. contributed reagents, materials and analysis tools. All authors discussed data and the manuscript. P.D.C. and F.L.M. wrote the manuscript.

**Funding**

This work was supported by the National Institutes of Health [R01GM089979 and start-up funds to F.L.M.; T32-GM007288 and 1F31NS083258 to P.D.C.]. Deposited in PMC for release after 12 months.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.124586/-/DC1

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