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 germline 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 translocation depend on kif5Ba, grip2a translocation 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; Illmsensee 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 zygotically accumulating GP, MTs are enriched in 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 zebrafish and Xenopus Buc/Velo interact with RNAhps 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...
Elinson, 1991; Jesuthasan and Strähle, 1997; Tran et al., 2012). In zebrafish, pMTA formation roughly coincides with asymmetric translocation of Syntabulin protein and wnt8a and grip2a RNAs (Ge et al., 2014; Lu et al., 2011; Nojima et al., 2010; Tran et al., 2012). Although maternal grip2a contributes to alignment and bundling of the pMTA (Ge et al., 2014), and vegetal MT plus-ends are oriented dorsalward (Tran et al., 2012), it remains unclear how pMTA rearrangements are initiated and which other factors are involved. Nevertheless, evidence from Xenopus, using antibodies and rigor mutants, implicates Kinesin-mediated transport of vegetal RNAs in oocytes (Gagnon et al., 2013; Messitt et al., 2008) and Kinesin-dependent pMTA formation (Marrari et al., 2000). Importantly, however, the specific Kinesin superfamily protein (Kif) involved is not known.

Here, we show that zebrafish maternal kif5Ba is essential for PGC specification and DV patterning. Our mutant analysis shows that maternal kif5Ba is dispensable for oocyte polarity, but acts in embryos to recruit GP to cleavage furrows, for PGC specification and for fertility. Moreover, Kif5Ba recruits or enriches Buc in cleavage furrows, where it probably mediates GP recruitment or stabilization, thus providing mechanistic insight into how GP is assembled at cleavage furrows. We further provide evidence for two mechanisms to asymmetrically distribute DDs in activated eggs: a kif5Ba-independent mechanism that mediates grip2a translocation, and a kif5Ba-dependent mechanism that organizes the pMTA and redistributes Syntabulin and wnt8a. These results provide mechanistic insight into Buc activities in embryos, PGC specification and the maternal mechanisms that generate DV asymmetry in early embryos.

RESULTS

Endogenous Buc localizes to the germ plasm of embryos and binds Kinesin-1

Buc is necessary and sufficient for GP recruitment in oocytes (Bontems et al., 2009; Heim et al., 2014; Marlow and Mullins, 2008), and, when overexpressed, Buc-GFP localizes to cleavage furrows in embryos (Bontems et al., 2009). However, it is unknown how Buc-GFP localizes to cleavage furrows and whether endogenous Buc does so, too. We examined endogenous Buc in cleavage-stage embryos using α-Buc antibodies (Heim et al., 2014) and found that endogenous Buc was enriched at distal cleavage furrows of wild-type (WT) embryos. These accumulations were cell cycle dependent, and were most apparent when chromosomes were metaphase embryos and n=1/18 anaphase embryos. To investigate further Buc recruitment to cleavage furrows, we performed a pull-down assay from ovary lysates using anti-Buc antibody and identified Kif5B as a binding partner by mass spectrometry (peptide threshold=95%; n=10 peptides; 7% coverage). To validate this interaction, we performed co-immunoprecipitation assays in HEK293T cells using GFP-Buc and HA-tagged Kif5Ba. Immunoprecipitation with anti-GFP showed that GFP-Buc, but not GFP, co-precipitated HA-Kif5Ba (Fig. 1C). To determine whether Kif5Ba and Buc colocalize in vivo we injected exogenous ha-kif5Ba and gfp-buc RNA into WT eggs. At the 16-cell stage, GFP-Buc was enriched in cleavage furrows as previously reported (Bontems et al., 2009), whereas HA-Kif5Ba was uniformly distributed (Fig. 1D-F). Together with our previous studies showing that kif5Ba is the main maternally expressed kif5 (Campbell and Marlow, 2013), these results indicate that Kif5Ba binds Buc and might mediate Buc localization in embryos.

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 F1 progeny and cDNA of homozygous mutants identified a 5 base pair (bp) deletion predicted to produce a premature stop codon (kif5Ba<sup>5-12</sup>) and a 6 bp deletion predicted to delete two amino acids (kif5Ba<sup>6-12</sup>) (Fig. 1H). qRT-PCR revealed nonsense-mediated decay of kif5Ba<sup>5-12</sup> but not of kif5Ba<sup>6-12</sup> transcripts in homozygous mutants (Fig. 1I). Despite mild craniofacial defects of kif5Ba<sup>5-12</sup> and kif5Ba<sup>6-12</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 Mki5fBa 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; Koprumer 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 Mki5fBa embryos (Fig. 2B,F); and thus, no PGCs were detected in Mki5fBa mutants (Fig. 2D,H). Conversely, vegetal localization of dazl RNA was intact in Mki5fBa 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 Mki5fBa 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 Mki5fBa mutants (Fig. 2N,O), indicating failed PGC specification. Consequently, Mki5fBa embryos developed exclusively as sterile males (Table 1) devoid of germ cells (Fig. 2P-Q′). 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 diverses (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). Together with our previous studies showing that kif5Ba is the main maternally expressed kif5 (Campbell and Marlow, 2013), these results indicate that Kif5Ba binds Buc and might mediate Buc localization in embryos.

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material Fig. S1I-L, O-R). Moreover, vasa and nanos3 were comparable in WT and kif5Ba−/− 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 Mki5Ba 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 is flanked by MTs oriented perpendicular to the furrow, known as a distal furrow-oriented V-like structure. Finally, furrow maturation, FMA enrichment resembles that of GP, and the furrow microtubule array (FMA), (Jesuthasan, 1998). During is flanked by MTs oriented perpendicular to the furrow, known as a distal furrow-oriented V-like structure. Finally, furrow maturation, FMA enrichment resembles that of GP, and the furrow microtubule array (FMA), (Jesuthasan, 1998). During

Providing exogenous kif5Ba to Mki5Ba mutants following fertilization rescues PGC specification

Because oocytes and cleavage furrows were intact, and GP components were available but not recruited in Mki5Ba embryos, we hypothesized that injection of kif5Ba RNA into Mki5Ba 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. 3H; 13.24±4.09 PGCs/side; n=21 embryos), Mki5Ba 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=21 embryos), and had no differences between WT and Mki5Ba embryos in the medial contractile band, the F-actin accumulations flanking the FMA, nor in the circumferential actin bands around the blastomeres (supplementary material Fig. S2A-F) (Theusch et al., 2006; Urven et al., 2006). β-tubulin staining confirmed that FMA MTs properly formed adjacent to the F-actin contractile band, with an intervening MT-free zone (supplementary material Fig. S2C-F), and angled MTs distally (supplementary material Fig. S2G,H). These results indicated that failed GP recruitment in Mki5Ba embryos was not due to furrow-associated cytoskeleton abnormalities.

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. Insets show DAPI. Arrowheads indicate Buc accumulation. (C) Schematic protein structure of Kif5Ba, illustrating motor, stalk and tail domains. Red dashed line indicates CRISPR target site. (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) 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. (H) qRT-PCR for kif5Ba expression in Mki5Ba embryos when chromosomes are decondensed (A), but not during metaphase (B) or anaphase. Insets show DAPI. 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 expression in Mki5Ba embryos when chromosomes are decondensed (A), but not during metaphase (B) or anaphase. Insets show DAPI. 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 expression in Mki5Ba embryos when chromosomes are decondensed (A), but not during metaphase (B) or anaphase. Insets show DAPI. 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 expression in Mki5Ba embryos when chromosomes are decondensed (A), but not during metaphase (B) or anaphase. Insets show DAPI. 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 expression in Mki5Ba embryos when chromosomes are decondensed (A), but not during metaphase (B) or anaphase. Insets show DAPI. Arrowheads indicate Buc accumulation.

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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 Mki5Ba 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 Mki5Ba 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,G′), reminiscent of M

grip2a

mutants (Ge et al., 2014). Because ventralization was only observed in progeny of homozygous kif5Ba

ae11/ae11

and kif5Ba

ae12/ae12

transheterozygous kif5Ba

ae11/ae12

females, but not WT or heterozygous genotypes, we concluded that kif5Ba mutation caused ventralization (Fig. 5H). kif5Ba

ae11/ae12

and kif5Ba

ae12/ae12

were more severe than kif5Ba

ae11/ae11

females, and had similar penetrance and expressivity. No phenotypes were observed in
heterozygotes for either allele. Together, these results are consistent with both kif5Ba<sup>ae11</sup> and kif5Ba<sup>ae12</sup> having LOF character, with kif5Ba<sup>ae12</sup> probably being a stronger LOF allele than kif5Ba<sup>ae11</sup>. Although unlikely, we cannot exclude the possibility that kif5Ba<sup>ae12</sup> 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 positioning Sybu in oocytes or during egg activation. Together, these results are consistent with roles for Kif5Ba in maintaining Sybu at the vegetal pole and is necessary for sybu RNA at the vegetal pole and is necessary for sybu RNA localization to occur. Syntubulin (Sybu) protein translocates after egg activation (Fig. 6O,P) (Nojima et al., 2010). In Mkif5Ba activated eggs, Sybu protein, like sybu RNA, was mislocalized in some Mkif5Ba eggs at 2 mpa, either aberrantly present on the lateral cortex or diffuse about the cortex (Fig. 6Q–R). Syntabulin (Sybu) protein translocates after egg activation and maintains kif5Ba<sup>ae12</sup> localization in Mkif5Ba activated eggs (Fig. 6L), but wnt8a RNA localization was not detected in Mkif5Ba activated eggs (Fig. 6N). These data suggest that kif5Ba maintains sybu RNA at the vegetal pole and is necessary for sybu RNA localization to occur.

Previous studies have shown that, like grip2a and wnt8a RNA, Syntubulin (Sybu) protein translocates after egg activation (Fig. 6O,P) (Nojima et al., 2010). In Mkif5Ba activated eggs, Sybu protein, like sybu RNA, was mislocalized in some Mkif5Ba eggs at 2 mpa, either aberrantly present on the lateral cortex or diffuse about the cortex (Fig. 6Q–R). Syntabulin (Sybu) protein translocates after egg activation and maintains kif5Ba<sup>ae12</sup> localization in Mkif5Ba activated eggs (Fig. 6L), but wnt8a RNA localization was not detected in Mkif5Ba activated eggs (Fig. 6N). These data suggest that kif5Ba maintains sybu RNA at the vegetal pole and is necessary for sybu RNA localization to occur.

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

Prior to asymmetric translocation of Sybu protein and wnt8a RNA (~20 mpa), the vegetal MT network reorganizes to form the vegetal pMTA defects we analyzed the MT network of kif5Ba<sup>ae12</sup> mutants, showing that MKIF5B is required for the proper organization of the vegetal MT network. To determine whether maternal Kif5Ba localizes maternal regulators of DV patterning, we performed in situ hybridization for sybu, grip2a and wnt8a RNA in eggs. As previously reported (Ge et al., 2014; Lu et al., 2011; Nojima et al., 2010), sybu, grip2a 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 translated in WT (Fig. 6C,H,L). In Mkif5Ba mutants, sybu RNA was initially vegetally localized (Fig. 6B), but was more dispersed [sybu domain angle in Mkif5Ba (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 Mkif5Ba mutants, although more Mkif5Ba mutants showed weak expression (Fig. 6D–F). qRT-PCR for sybu RNA confirmed that it was significantly elevated in Mkif5Ba 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 Mkif5Ba mutants. Unlike sybu RNA, in Mkif5Ba 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 Mkif5Ba 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 sybu RNA localization to occur.

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Previous studies have shown that, like grip2a and wnt8a RNA, Syntubulin (Sybu) protein translocates after egg activation (Fig. 6O,P) (Nojima et al., 2010). In Mkif5Ba activated eggs, Syb protein, like sybu RNA, was mislocalized in some Mkif5Ba eggs at 2 mpa, either aberrantly present on the lateral cortex or diffuse about the cortex (Fig. 6Q–R). Syb protein was vegetally localized in n = 3/12 Mkif5Ba<sup>ae11</sup> (n = 4/9) Mkif5Ba<sup>ae11</sup> and n = 2/9 Mkif5Ba<sup>ae12</sup> compared with n = 13/15 WT eggs. At 45 mpa, in a subset of eggs, Syb either failed to translocate or was not detectable (Fig. 6R–T; Syb translocated normally in n = 11/11 Mkif5Ba<sup>ae11</sup> and n = 3/11 for Mkif5Ba<sup>ae12</sup>). 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 Kif5Ba 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 Mkif5Ba ventralization phenotype by injecting ha-kif5Ba RNA into Mkif5Ba embryos; however, this did not suppress the penetrance or expressivity of Mkif5Ba 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.
activated eggs. Surprisingly, although pMTA formation was robust in WT (Fig. 7A,M), it failed in most M\textit{kif5Ba} activated eggs (Fig. 7D, M). Instead, M\textit{kif5Ba} vegetal MTs either appeared bundled and randomly oriented (Fig. 7G,M) or non-bundled with a crosshatch appearance (Fig. 7J,M). No differences were observed between the lateral cortex MT networks of WT and M\textit{kif5Ba} 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 M\textit{kif5Ba} mutants, CGs were labeled with \textit{Maclura pomifera} agglutinin (MPA) lectin (Becker and Hart, 1996, 1999). M\textit{kif5Ba} 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 M\textit{kif5Ba} mutants, regardless of pMTA defect severity (Fig. 7M).

Specifically, M\textit{kif5Ba} mutants with severe CG exocytosis deficits but normal vegetal pMTAs and M\textit{kif5Ba} 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 M\textit{kif5Ba}\textsubscript{ae12/ae12} mutants than M\textit{kif5Ba}ae11/ae11 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 \textit{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 assemblies 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 \textit{wnt8a}, and that asymmetry of \textit{grip2a} occurs independently of \textit{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; Koprunner 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 \textit{kif5Ba} mutant ovaries, our ha-\textit{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 M:kif5Ba 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 RNAbps (Heim et al., 2014), it might recruit GP-RNPs indirectly, although we cannot exclude the possibility that Buc possesses uncharacterized RNA binding elements.

Fig. 6. Maternal kif5Ba localizes dorsal determination components in activated eggs. (A–D) Sybu is vegetally localized at 2 mpa (A,B) and at 45 mpa (C,D) in both WT (A,C) and M:kif5Ba mutant (B,D) activated eggs. However, sybu is more dispersed at 2 mpa (B) and appears less abundant in most M:kif5Ba at 45 mpa (D,D'). [2 mpa: n=30/30 for M:kif5Ba2n=172 uninjected and N=2 females), and at 45 mpa: quantification in panels.] (E) qRT-PCR reveals significantly more abundant sybu RNA in M:kif5Ba mutants than in M:kif5Ba at 2 mpa, although this RNA excess is not maintained at 45 mpa. Error bars show mean±s.d.; Student's t-test, *P=0.0219. (F–I) grip2a is vegetally localized at 2 mpa (F,G) and shifts asymmetrically by 45 mpa (H,I) in both WT (F,H) and M:kif5Ba mutant (G,I) activated eggs. [2 mpa: n=37/37 for M:kif5Ba2n=172 (N=2 females), and at 45 mpa: see N for quantification.] (J–M) Wnt8a is vegetally localized at 2 mpa in both WT (J) and M:kif5Ba mutant (K) activated eggs. However, wnt8a is asymmetric at 45 mpa in WT (L) but not M:kif5Ba mutant (M) activated eggs. [2 mpa: n=24/24 for M:kif5Ba2n=172 (N=2 females), n=29/29 for WT (N=2 females) have vegetally localized wnt8a; 45 mpa: see N for quantification.] (N) Fraction of eggs exhibiting dorsally activated at 2 mpa (O–P) and at 45 mpa (Q–R). See text for quantification. (S) Injection of ha-kif5Ba RNA into 1-cell-stage M:kif5Ba mutants fails to suppress DV patterning defects. [n=138 un.injected and n=81 injected M:kif5Ba2n=172 (N=1 female), n=172 uninjected and n=80 injected M:kif5Ba2n=172 (N=2 females)]. Arrowheads designate the lateral limits of each expression domain.
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.

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Dorsal Specification

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<tr>
<th></th>
<th>Wildtype</th>
<th>Maternal kif5Ba mutant</th>
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<tbody>
<tr>
<td>DDs</td>
<td>symmetric at vegetal</td>
<td>shift towards dorsal</td>
</tr>
<tr>
<td>1 cell</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>V</td>
<td>Organize MTs</td>
<td>Parallel MTs</td>
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<td>D</td>
<td>Transports/Tethers</td>
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<td></td>
<td>RNP-Buc-Adaptors</td>
<td></td>
</tr>
<tr>
<td>4 cell</td>
<td>4</td>
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</tr>
<tr>
<td></td>
<td>Proper DV axis</td>
<td>Ventralized + No PGCs</td>
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<tr>
<td></td>
<td>PGCs intact</td>
<td>Sterile Male Adult Escapers</td>
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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 was 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: TAGGACAGGATAGGCGTGAT and gRNA-R: AAAACATCGACGGCTATCCGTG 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 MssI-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>oe11</sup> and kif5Ba<sup>ae12</sup> mutations was amplified with primers 5'-GGAGTGCACCATT-AAAAGTCATGTG and 5'-GTCGGTGCATAATATGAGGTC. Restriction enzyme (RE) digestion with PvuI for kif5Ba<sup>oe11</sup> produced two smaller fragments for the wild-type allele but not the mutant allele. RE digestion with MboI 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.
overnight. RNAscope Blank C1 probe, Dr-nanos3-C2 probe (ACD Bio, 404521-C2) and Dr-vasa-C3 probe (ACD Bio, 407271-C3) were used.

**Immunofluorescence and F-actin, cortical granule and DiOC6 labeling**

For whole-mount IF of microtubules, F-actin and Buc, eggs and embryos were fixed with microtubule staining buffer (80 mM K-PIPES pH 6.8, 5 mM EGTA, 1 mM MgCl2, 3.7% formaldehyde, 0.25% glutaraldehyde, 0.2% Triton X-100) for 4-5 h at RT. Staining was performed immediately. For confocal imaging, embryos or ovaries were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated in MeOH and placed at −20°C. Anti-β-tubulin (Millipore, MAB3408) was diluted at 1:100, Anti-Bucky ball y1165 at 1:500 (Heim et al., 2014), Anti-Syntabulin Syn1 at 1:1000 (Nojima et al., 2010), Anti-Vasa at 1:5000 (Knaut et al., 2000) and Anti-HA at 1:500 (Roche, 11867423001). Alexa Fluor 488 and Alexa Fluor 568 (Molecular Probes) secondary antibodies were diluted at 1:50. CGs were counted in a defined vegetal region at depth×height×width of 4 µm×59.45 µm×59.45 µm. F-actin was stained by incubating with Rhodamine-Phalloidin (Life Technologies, AM1340).

**RT-PCR**

Total RNA was extracted from pooled embryos (n=20-30/stage) using Trizol (Life Technologies, 15596). cDNA was prepared with SuperScript III Reverse Transcription Kit (Life Technologies, 18080-051). RT-PCR was performed using the following primers: ef1α (Fig. 1) and vasa: (Heim et al., 2014); dazl and nanos3 (Hartung et al., 2014); kif5Ba: 5'-GACCTTGCA-CAACCTCAGGAAA and 5'-GCAACGCTTCGTTCCAGT; efta (Fig. 6): 5'-CTGGTTCAGGAGGATGGAAGA and 5'-CTGGTTCAGGAAGGATGGAAGA; 5'-GGAAAGTCAAAGACCCGAGAAA and 5'-TGGATGCCATGATGTTGCACCA. qRT-PCR reactions were performed in triplicate with SYBR Green Master Mix (Thermo Scientific) and an Eppendorf realplex Mastercycler. Expression relative to WT was quantified with the ΔΔCt method using efta as the standard gene.

**Histology**

Females or males were anesthetized in Tricaine as described (Westereifel, 2000), and the ovaries or testes were dissected and fixed in 4% paraformaldehyde overnight at 4°C. Fixed ovaries were washed in PBS, dehydrated in methanol, embedded in paraffin and sectioned. Deparaffinized slides were stained in H&E, coated with Permount solution (Fisher Scientific), coverslipped and imaged. Oocytes were staged according to Selman et al. (1993).

**RNA injection**

ha-kif5Ba RNA was transcribed from pCS2+HA-Kif5Ba (Nojima et al., 2010) with the mMESSAGE mMACHINE SP6 Transcription Kit (Life Technologies, AM1340). pCS-GFP-Buc was created by recombining pCR8-chuc80 (Heim et al., 2014) with pCSGFPDest (Villefranc et al., 2007) (Invitrogen). gfp-buc RNA was transcribed from pCS-GFP-Buc with the mMESSAGE mMACHINE SP6 Transcription Kit. cherry-24xMBS RNA was used as a control and prepared as previously described (Campbell et al., 2015). Embryos from kif5Ba+/− females were collected within 15 min of spawning. Approximately 1 nl of 500-800 ng/µl (ha-kif5Ba or cherry-MBS) or 400 ng/µl (gfp-buc) injection solution was injected into 1-cell-stage embryos.

**Buc pull-down**

To reduce yolk content, ovaries were dissected from Buc pull-down (Nippara et al., 2014); Anti-Syntabulin Syn1 at 1:1000 (Nojima et al., 2010), Anti-Bucky ball y1165 at 1:500 (Heim et al., 2014), Anti-vasa-C3 probe (ACD Bio, 404521-C2) and Dr-vasa-C3 probe (ACD Bio, 407271-C3) were used. To reduce yolk content, ovaries were dissected from Buc pull-down (Nippara et al., 2014); Anti-Syntabulin Syn1 at 1:1000 (Nojima et al., 2010), Anti-Bucky ball y1165 at 1:500 (Heim et al., 2014), Anti-vasa-C3 probe (ACD Bio, 404521-C2) and Dr-vasa-C3 probe (ACD Bio, 407271-C3) were used.
Jesuthasan, S. and Stra (2015) This is an example of a double author name.


