A novel isoform of *Drosophila* non-muscle Tropomyosin interacts with Kinesin-1 and functions in mRNA localization.

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Abstract:
Recent studies have revealed that diverse cell types use mRNA localization as a means to establish polarity. Despite the prevalence of this phenomenon, much less is known regarding the mechanism by which mRNAs are localized. The *Drosophila* oocyte provides a useful model for examining the process of mRNA localization. *oskar* (*osk*) mRNA is localized at the posterior of the oocyte, thus restricting the expression of Oskar protein to this site. The localization of *osk* mRNA is microtubule-dependent and requires the plus-end directed motor, Kinesin-1. Unlike most Kinesin-1 cargoes, localization of *osk* mRNA requires the Kinesin heavy chain (Khc) motor subunit, but not the Kinesin light chain (Klc) adaptor. In this report, we demonstrate that a novel isoform of Tropomyosin, referred to as Tm1C, directly interacts with Khc and functions in concert with this microtubule motor to localize *osk* mRNA. In contrast to *osk*, several additional Khc-dependent processes in the oocyte are unaffected upon loss of Tm1C. Our results therefore suggest that the Tm1C-Khc interaction is specific for the *osk* localization pathway.
Introduction:
Asymmetric protein sorting underlies the establishment and maintenance of cell polarity. Recent studies have revealed that mRNA localization is a prevalent mechanism used by different cell types for restricting protein localization (Cajigas et al., 2012; Jambor et al., 2015; Lecuyer et al., 2007; Zivraj et al., 2010). Thus, understanding the mechanism of mRNA localization is critical to gaining a deeper understanding of cell polarity establishment.

Although examples of localized mRNAs have been identified in a variety of organisms, mechanistic studies are easier to address using simpler model systems. For example, localization of ASH1 mRNA in the yeast, Saccharomyces cerevisiae, represents the best-understood example of a localized transcript (Gonsalvez et al., 2005). In this system, ASH1 mRNA is first identified by the RNA binding protein, She2p (Niedner et al., 2014). This complex is then coupled to the Myo4p motor via the adaptor She3p (Singer-Kruger and Jansen, 2014). In addition, the various cis-elements within ASH1 that mediate localization have also been identified (Chartrand et al., 1999).

In Drosophila blastoderm embryos, several mRNAs are localized to the apical surface. This process is dependent on the minus-end directed microtubule motor, Dynein (Bullock, 2011). These mRNAs are thought to be identified by the RNA binding protein, Eaglitarian (Egl) (Dienstbier et al., 2009). The Egl/mRNP complex is then linked to Dynein via the Egl-Dynein light chain (Dlc) interaction or via the Egl binding protein, Bicaudal D (BicD) (Navarro et al., 2004). In mammalian cells, BicD has been shown to bind the Dynein regulator, Dynactin (Hoogenraad et al., 2001). However, even prior to the blastoderm stage, polarity is already established by events that take place in the oocyte.

The localization of oskar (osk), bicoid (bcd) and gurken (grk) mRNAs is required for establishing the polarity of the oocyte and future embryo (St Johnston, 2005). bcd and grk mRNAs localize at the anterior and dorsal-anterior regions of the oocyte respectively (Berleth et al., 1988; Neuman-Silberberg and Schupbach, 1993). Their localization is thought to depend primarily on Dynein (MacDougall et al., 2003; Schnorrer et al., 2000). Although the exact link between these mRNAs and Dynein has not been identified, the available evidence suggests that this role is performed by Egl (Dienstbier et al., 2009; Sanghavi et al., 2016).

osk mRNA localizes at the posterior of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991). The primary motor responsible for this localization is the plus-end directed microtubule motor, Kinesin-1 (Brendza et al., 2000). However, recent findings suggest that Dynein is also required for efficient osk localization (Sanghavi et al., 2013). Kinesin-1 typically consists of a Kinesin heavy chain (Khc) motor subunit and a Kinesin light chain (Klc) adaptor (Hirokawa et al., 2009). Thus, most cargoes that are transported by Kinesin-1 require both Khc and Klc. However, over the past several years, numerous cargoes have been identified that are transported by Kinesin-1 in a Klc independent manner (Hirokawa et al., 2009). In these cases, Khc typically associates with cargo using an alternative adaptor. It appears that the localization of osk might involve such an alternative adaptor. Although osk is completely delocalized in khc mutants, it remain localized in klc nulls (Palacios and St Johnston, 2002).
In order to better understand the mechanism by which Khc functions in osk mRNA localization, we purified the motor and identified interacting partners using mass spectrometry. Using this approach, we identified a novel isoform of Tropomyosin, Tm1C, as an interacting partner of Khc. In this report, we demonstrate that Tm1C functions in concert with Khc to localize osk mRNA at the posterior pole. However, several additional Khc processes appear to function independently of Tm1C. Finally, we demonstrate that Tm1C expression is not restricted to the female germline. This raises the intriguing possibility that this novel isoform of tropomyosin might have functions in addition to osk localization.
Results:
Tropomyosin 1C (Tm1C) is an interacting partner of Kinesin heavy chain.
The mechanism by which Khc localizes osk mRNA is unknown, but it is assumed that an
adaptor other than Klc functions in this process. In order to identify proteins that associate
with Khc, we undertook a proteomic purification strategy. Ovarian lysates were prepared
from strains expressing either GFP or Khc-GFP. The tagged proteins were
immunoprecipitated, the co-precipitating proteins were eluted and identified using mass
spectrometry. The entire experiment was performed twice.

Peptides corresponding to Klc were highly enriched in Khc-GFP pellets (Table 1). Thus,
even though several Kinesin-1 cargoes in the oocyte are localized independent of Klc,
the light chain remains abundantly associated with Khc in vivo. Excluding typical
contaminants, the next most abundant protein in Khc-GFP pellets corresponded to
Tropomyosin1 (Tm1) (Table 1).

Tropomyosins are actin binding proteins that have a well-documented role within muscle
in mediating contraction (Gunning et al., 2015). However, non-muscle isoforms are also
expressed and these function in a variety of actin-dependent processes (Manstein and
Mulvihill, 2016). Drosophila contains two tropomyosin genes, tropomyosin1 (tm1) and
tropomyosin2 (tm2). Although most publications simply refer to these genes as tm1 and
tm2, genome annotation suggests that this family is capable of expressing numerous
isoforms (Supplemental Fig. 1A). In fact, a recent publication by Goins and Mullins
demonstrated that Drosophila S2 cells express two isoforms of Tm1 (Tm1A and Tm1J)
and one isoform of Tm2 (Tm2A) (Goins and Mullins, 2015).

In order to determine which isoform of Tm1 associated with Khc, we analyzed peptide
sequences obtained in Khc-GFP pellets. Each Tm1 peptide could be mapped to Tm1C,
Tm1H, and Tm1I (Supplemental Fig. 1B). Tm1C and Tm1I differ in their 5’UTR, but
encode identical proteins (Fig. 1A). By contrast, Tm1H is predicted to contain an amino
terminal extension not found in Tm1C and Tm1I (Fig. 1A). Peptides corresponding to this
region were not recovered in Khc-GFP pellets. Thus, the most likely interacting partner
for Khc is either Tm1C or Tm1I. Because these isoforms encode identical proteins, for
the sake of simplicity, we will refer to the potential Khc-interacting isoform as Tm1C.

As a first step towards validating the Khc/Tm1C interaction, we immunoprecipitated Khc-GFP
from ovarian lysates and examined the pellet using an antibody against endogenous Tm1C. The
antibody was generated using peptide sequences found in both Tm1C and Tm1J, and is therefore
capable of detecting both isoforms. Consistent with our proteomic results, endogenous Tm1C, but
not Tm1J, co-immunoprecipitated with Khc-GFP (Supplemental Fig. 1C). Using a complementary
approach, we generated transgenic flies expressing GFP-Tm1C driven by the vasa promoter (Sano
et al., 2002). As expected, endogenous Khc specifically co-precipitated with GFP-Tm1C
(Supplemental Fig. 1D).
In order to further characterize the Khc/Tm1C interaction, we expressed tagged versions of these proteins in *Drosophila* S2 cells and performed a co-immunoprecipitation experiment. Consistent with the ovary purification result, RFP-Khc specifically co-precipitated with GFP-Tm1C (Fig. 1B). For this experiment, a version of Khc lacking the N-terminal motor domain was used. However, a full-length RFP-Khc construct containing the motor domain is also able to bind GFP-Tm1C (data not shown). In contrast to GFP-Tm1C, RFP-Khc did not co-precipitate with GFP-Tm1A, Tm1J or Tm2A (Fig. 1C). Thus, the interaction between Khc and Tropomyosin is specific for the Tm1C isoform. We further demonstrate that purified GST-Tm1C produced in bacteria was able to interact with recombinant RFP-Khc produced using coupled in vitro transcription and translation (Fig. 1D). Thus, the interaction between Khc and Tm1C is direct.

Most tropomyosins, including those found in mammals, are relatively small, consisting of fewer than 300 amino acids. Their structure consists of alpha-helical coiled-coil domains suited for binding along the sides of actin filaments (Gunning et al., 2005). These features are shared by Tm1A, Tm1J, and Tm2A (Fig. 1E and data not shown). By contrast, at 441 amino acids, Tm1C represents a very large tropomyosin. In addition, Tm1C contains a long N-terminal extension that is not predicted to form a coiled-coil (Fig. 1E). Because this N-terminal extension is unique to Tm1C, we predicted that this region would be involved in Khc binding. Unexpectedly, a Tm1 construct containing the C-terminal region was able to interact more strongly with Khc than the construct containing the N-terminal region (Fig. 1E). Thus, even though Khc specifically interacts with the unique Tm1C isoform, the residues that are most critical for binding comprise the conserved coiled-coil domain.

**Tm1C interacts with a C-terminal domain of Khc.**

Experiments in the preceding section revealed that the motor domain of Khc (Fig. 2A, red box) was not required for binding Tm1C. In addition to the motor domain, Khc also contains coiled-coil domains required for dimerization of the heavy chain (Fig. 2A, green boxes). A smaller domain C-terminal to these is involved in binding Klc (Fig. 2A, yellow box). Certain adaptors, such as the mitochondrial adaptor Milton, bind to the same region of Khc as does the light chain, thus competing for binding with Klc (Glater et al., 2006).

In order to determine the Tm1C binding site within Khc, we generated several RFP-tagged C-terminal Khc truncations. These truncations were co-transfected into S2 cells along with full-length GFP-Tm1C and binding was determined using co-immunoprecipitation. A Khc construct missing the auto-inhibitory domain was able to bind Tm1C (Fig. 2B, lane 2). However, further truncation beyond this region compromised the Khc-Tm1C interaction (Fig. 2B, lanes 3-5). The same Khc truncations were co-transfected into S2 cell along with full-length GFP-Klc and binding was determined using co-immunoprecipitation. Klc was able to interact with a subset of these truncations (Fig. 2C, lanes 1-3). However, as expected, deletion of the Klc binding site disrupted the Khc-Klc interaction (Fig. 2C, lane 4). Consistent with these findings, a small Khc construct containing residues 824 to 975 was able to interact with Tm1C but not Klc (Fig. 2D). These results suggest that Tm1C and Klc bind to different regions of Khc. Furthermore, the binding site for Tm1C lies between residues 850 and 941.

In order to more precisely map the region of Tm1C binding, we created small deletions within full length Khc. These internally deleted RFP-Khc constructs were transfected into S2
cells along with full-length GFP-Tm1C and binding was determined using co-immunoprecipitation. Deletions spanning residues 849 to 887 had a minimal effect on Tm1C binding (Fig. 2E, lanes 1-3). This region was of interest because previous studies have shown it to function as a cargo recognition domain in *Neurospora crassa* (Fig. 2A, light blue box) (Seiler et al., 2000). By contrast, deletion of amino acids 914 to 936 resulted in almost complete loss of binding (Fig. 2A dark blue oval, Fig. 2E lane 4). Thus, a region within Khc which has been shown to bind microtubules in vitro (Andrews et al., 1993; Hackney and Stock, 2000), is the primary binding site for Tm1C.

The alignment in Fig. 2A illustrates the high degree of conservation between *Drosophila* Khc and its mammalian homolog, Kif5b, within the Tm1C binding region. Interestingly, in vivo truncation studies have identified this region of Khc as being required for osk localization (Williams et al., 2014). However, a potential adaptor or cargo that binds this region of Khc has thus far not been identified.

**Localization of Tm1C.**

As noted in the preceding section, we generated an antibody against Tm1C. Although our antibody was capable of detecting Tm1C on western blots, we were unable to detect specific signal using immunofluorescence, thus limiting its use for localization studies. We therefore made use of a strain expressing GFP-Tm1C, in which expression of the fusion protein was driven by *vasa* regulatory elements (Sano et al., 2002).

In early stage egg chambers, GFP-Tm1C was highly enriched in the oocyte (Fig. 3A). GFP-Tm1C also displayed perinuclear enrichment consistent with the nuage (Fig. 3A, Supplemental Fig. 2A). The nuage is an electron-dense region that surrounds nurse cell nuclei. In stage 8 egg chambers, GFP-Tm1C could be detected at the anterior corners of the oocyte as well as the posterior (Fig. 3B, arrow and arrowhead respectively). A similar localization was observed in stage 9 egg chambers (Fig. 3C). By stage 10, the nuage localization of GFP-Tm1C persisted, and within the oocyte, GFP-Tm1C localized around the cortex (Fig. 3D).

In order to validate the localization pattern of GFP-Tm1C, a strain expressing unfused GFP in the germline was processed in a similar manner. Although unfused GFP was not specifically enriched in the oocyte and did not localize to the nuage, it could be detected around the oocyte cortex (Supplemental Fig. 2B and data not shown). Based on this result, we are not able to conclude whether the cortical localization of GFP-Tm1C is a specific property of Tm1C, or whether it is an artifact of the GFP-Tm1C fusion protein.

Consistent with the protein-protein interaction result, a significant degree of co-localization was observed between GFP-Tm1C and Khc (Figs. 3E, F). A few differences are worth noting, however. The oocyte enrichment and nuage localization was more evident for GFP-Tm1C in comparison to Khc (Fig. 3E). Furthermore, in stage 10 egg chambers, the posterior enrichment was more apparent for Khc in comparison to GFP-Tm1C (Fig. 3F', arrow).

Within egg chambers, filamentous actin (F-actin) is highly enriched around the cortex and within ring canals. Given that Tropomyosins are actin-binding proteins, it was surprising that minimal co-localization was observed between GFP-Tm1C and F-actin (Figs. 3G, H).
In order to test the dependence of GFP-Tm1C localization on F-actin, we treated egg chambers with LatrunculinA, a drug that destabilizes F-actin. Although F-actin staining was virtually eliminated by LatrunculinA treatment, the localization of GFP-Tm1C appeared unaffected (Supplemental Fig. 2C-F).

We next determined whether the localization of GFP-Tm1C was sensitive to the presence of Khc. In order to deplete Khc, we expressed an shRNA against *khc* using a germline driver (Sanghavi et al., 2016). As expected, expression of this shRNA resulted in significant depletion of Khc (Supplemental Fig. 3A). Unexpectedly, the germline level of GFP-Tm1C was also greatly reduced in these flies. This result is specific to Khc depletion. GFP-Tm1C levels were unaffected in strains expressing a control shRNA (Fig. 3I-K). Similar results were obtained by examining ovarian lysates from these strains using western-blot (Supplemental Fig. 3B). It should be noted however, that because these lysates were prepared using whole ovaries, western analysis underestimates the true level of depletion in stage 10 egg chamber. Khc is not depleted in follicle cells or in early-stage egg chambers, both of which are present in whole ovary lysates.

As shown using *khc* nulls (Januschke et al., 2002; Mische et al., 2007), depletion of Khc results in formation of numerous actin foci within the oocyte (Fig. 3J). Despite using a high-gain setting, we were not able to detect co-localization between the residual GFP-Tm1C and these foci (Fig. 3J). Thus, the localization and expression level of GFP-Tm1C in egg chambers correlates to a greater degree with the presence of Khc than with F-actin.

**Tm1C depletion results in osk delocalization.**

In order to determine whether Tm1C is required for *osk* localization, we designed shRNA constructs that were specific for this isoform. The shRNAs were expressed using a previously characterized germline driver (Sanghavi et al., 2016). Ovarian lysates were prepared from these flies and examined by blotting using our Tm1 antibody. Although tm1 shRNA-1 and tm1 shRNA-2 were capable of depleting Tm1C, tm1 shRNA-2 consistently displayed greater depletion (Fig. 4A). In contrast to Tm1C, neither shRNA depleted Tm1J.

We next examined the localization of *osk*. *osk* mRNA was visualized using single molecule fluorescent in situ hybridization (smFISH) (Little et al., 2015; Little et al., 2013). In this approach, the target mRNA is examined using 48 unique DNA oligonucleotide probes that are directly conjugated to a fluorochrome. smFISH enables detection of mRNA with a much higher sensitivity and specificity in comparison to traditional methods. As expected, *osk* mRNA was highly enriched at the posterior pole in egg chambers expressing a control shRNA (Fig. 4B, E-G). As a control for specificity, we examined egg chambers using smFISH probes against *gapdh1* and 2 (*gapdh1/2*). Higher levels of *gapdh* mRNA was observed in nurse cells in comparison to the oocyte, and no enrichment was detected at the posterior pole (Supplemental Fig. 3C).

*osk* mRNA was still enriched at the posterior in egg chambers expressing the milder shRNA, tm1 shRNA-1 (Fig. 4C, E). However, a significant fraction of egg chambers also contained delocalized signal close to the posterior pole (Fig. 4C, E). By contrast, *osk* mRNA was almost completely delocalized around the cortex in egg chambers expressing the more potent tm1 shRNA-2 (Fig. 4D, E). A similar localization was observed for the
osk mRNP component, Staufen, in Tm1C depleted egg chambers (Supplemental Fig. 3D-F) (Zimyanin et al., 2008).

Translation of osk mRNA is regulated by several factors to ensure that only localized transcripts are translated (Kugler and Lasko, 2009). Thus, delocalization of osk mRNA often results in greatly reduced expression of Osk protein. As expected, Osk protein could not be readily detected in Tm1C depleted egg chambers (Supplemental Fig. 3G, H). Osk protein is required for recruiting pole plasm components such as Vasa (Vas) to the posterior pole. Consistent with reduced Osk protein expression, Vasa was no longer enriched at the posterior pole in Tm1C depleted oocytes (Supplemental Fig. 3G', H').

To demonstrate the specificity of this phenotype, the GFP-Tm1C construct used in the preceding section was brought into the background of tm1 shRNA-2. The tm1C coding sequence in this construct was mutated such that it was no longer recognized by the shRNA, yet still encoded wild-type protein. Thus, these flies will be depleted of endogenous Tm1C, but will express transgenic GFP-Tm1C. osk mRNA and pole plasm components were correctly localized to the posterior pole in these flies (Fig. 4E, Supplemental Fig. 3I-K). We therefore conclude that these phenotypes are specific to Tm1C depletion.

A recent analysis of osk using smFISH revealed that the mRNA undergoes assembly into large particles (Little et al., 2015). This occurs in a step-wise fashion. Upon entry into the oocyte, osk particles coalesce and increase in size (Little et al., 2015). Subsequently, during transit to the posterior, osk mRNA oligomerizes further to generate larger particles (Little et al., 2015). We obtained similar results using egg chambers expressing a control shRNA (Fig. 4F', F''). This phenotype was specific to osk and was not observed for gapdh mRNA (Supplemental Fig. 3C).

High resolution imaging of stage 10 egg chambers expressing tm1 shRNA-2 revealed that osk retained the ability to coalesce into large particles upon entry into the oocyte (Fig. 4H, H'). In addition, large osk particles could also be visualized close to the posterior and lateral cortex (Fig. 4H'', H'''). In fact, these particles were easier to observe in Tm1C depleted egg chambers (Fig. 4F vs 4H). This result is expected because in control egg chambers the majority of osk particles are anchored at the posterior pole. By contrast, in Tm1C depleted oocytes, the bulk of osk is present in delocalized particles. In late stage egg chambers, osk particles could be detected around the cortex of Tm1C depleted oocytes, but was restricted to the posterior in controls (Fig. 4G, I). Thus, Tm1C is required for localization of osk mRNA at the oocyte posterior, but is dispensable for assembly of osk into oligomeric particles.

We next examined the localization of osk in Khc depleted egg chambers. Loss of Khc has been shown to result in osk mRNA delocalization (Brendza et al., 2000). However, these mutants have not been examined using smFISH. Consistent with Tm1C functioning in concert with Khc in the osk localization pathway, depletion of Khc resulted in mislocalization of osk mRNA around the oocyte cortex (Fig. 4J, K). As with Tm1C depletion, oligomerization of osk was not affected upon loss of Khc (Fig. 4J', J'', J''').

A different phenotype was observed in Egl depleted egg chambers. Egl is thought to function as an adaptor for Dynein (Navarro et al., 2004), and has been shown to link Dynein to localized transcripts in the embryo (Dienstbier et al., 2009). In Egl depleted egg chambers, osk mRNA was diffusely distributed (Fig. 4L). A significant accumulation of
osk was detected in nurse cells (Fig. 4L'). This is consistent with the role of Dynein in transporting osk mRNA from nurse cells into the oocyte (Clark et al., 2007; Mische et al., 2007). As a consequence, the oligomerization of osk into larger particles upon oocyte entry was less obvious in Egl depleted egg chambers (Fig. 4L''). Furthermore, osk particles in the posterior region of Egl depleted egg chambers were smaller than those observed in controls (Fig. 4L''''). Thus, in contrast to Tm1C and Khc, Egl appears to be required for efficient transport of osk into the oocyte and for the formation of oligomeric particles.

**Tm1C is not required for all Kinesin processes.**

Apart from osk, khc nulls are also associated with delocalization of the oocyte nucleus, aberrant formation of actin spheres, and delocalization of *gurken* (grk) and *bicoid* (bcd) mRNAs (Brendza et al., 2002; Duncan and Warrior, 2002; Januschke et al., 2002). shRNA-mediated depletion of Khc produced a similar range of phenotypes (Fig. 5A-L). In contrast, the oocyte nucleus was correctly localized in Tm1C depleted egg chambers (Fig. 5F). Additionally, actin spheres were not observed and grk and bcd mRNAs remained correctly localized (Fig. 5C, I, L). Thus, Tm1C appears to be required for only a subset of Khc-dependent functions.

Several mRNAs including *nanos* (nos) and *cyclinB* (cycB) are localized at the posterior of late stage egg chambers and embryos (Dalby and Glover, 1992; Gavis and Lehmann, 1992). The localization of these mRNAs requires Osk protein. Consistent with the reduced expression of Osk in Tm1C depleted egg chambers, early-stage embryos from these mothers contained delocalized nos and cycB mRNA (Fig. 5M-P). As expected, given the defect in pole plasm formation, blastoderm-stage embryos from these mothers failed to form primordial germ cells (Fig. 5N', P').

**Generation of a Tm1C null.**

In the preceding section, we examined the role of Tm1C in osk mRNA localization using shRNA-mediated depletion. In order to independently validate this finding, and to determine whether Tm1C has roles outside of the germline, we sought to create an isoform specific null. In order to create this null, we chose to delete a small region between the 5'UTR and the first exon of Tm1C and I isoforms (Fig. 6A). This removes the initiating ATG codon, changes the reading frame, and results in several premature stop codons. We used a recently described CRISPR/Cas9 approach to delete this region (Gratz et al., 2014). In this strategy, two independent guide RNAs are designed to cleave at desired loci, and the intervening sequences are replaced using homology directed repair (Gratz et al., 2014). We refer to this mutant as tm1_delC.

tm1_delC homozygotes are semi-viable. Approximately 20% of mutants die during pupation. Ovarian lysates were prepared from homozygous and wild-type adult females and analyzed by western blotting. Confirming the status of this mutant as a true null, the band corresponding to Tm1C was absent in mutant lysates (Fig. 6B). However, the levels of Tm1J and Khc were unaffected (Fig. 6B).

We next examined the localization of osk mRNA in tm1_delC females (Fig. 6C, D). As with shRNA-mediated depletion, complete loss of Tm1C resulted in delocalization of osk
mRNA around the oocyte cortex (Fig. 6D). However, transport of osk into the oocyte and oligomerization into large particles was not affected (Fig. 6D). Furthermore, expression of GFP-Tm1C in the tm1_delC background completely restored posterior osk mRNA localization (Fig. 6E).

Consistent with Tm1C depletion, grk mRNA was correctly localized in tm1_delC mutants (Supplemental Fig. 4A). Furthermore, the oocyte nucleus was correctly positioned and aberrant actin spheres were not observed (Supplemental Fig. 4B). Lastly, although the pole plasm component Vasa was delocalized in tm1_delC nulls, (Fig. 6F, G), Khc and Dhc remained enriched at the posterior pole (Fig. 6H, I). Thus, in the absence of Tm1C, Khc retains the ability to transport Dhc to the oocyte posterior.

The partial lethality associated with tm1_delC nulls prompted us to examine whether this unique Tropomyosin isoform was expressed outside of the female germline. Consistent with this notion, Tm1C could be detected in lysates prepared from male flies (Supplemental Fig. 4C). These findings are supported by published RNA-seq results from Gravely et al., (Graveley et al., 2011) (Supplemental Fig. 4D). However, osk expression is restricted to the female germline (Supplemental Fig. 4D). This raises the intriguing possibility that Tm1C might function along with Khc to facilitate cargo transport in additional tissues. Further studies are needed to fully examine the organismal roles of Tm1C.
Discussion: Localization of osk mRNA at the posterior of Drosophila oocytes requires Khc, but is independent of the canonical Kinesin-1 adaptor, Klc (Brendza et al., 2000; Palacios and St Johnston, 2002). In this report, we demonstrate that a novel isoform of Tropomyosin, referred to as Tm1C, directly interacts with Khc and functions in concert with the motor to localize osk mRNA.

Tropomyosins are actin-binding proteins that are critical for muscle contraction (Gunning et al., 2005). However, non-muscle isoforms are also expressed and these isoforms contribute to a variety of actin-dependent processes (Gunning et al., 2005). Mammalian genomes contain 4 tropomyosin genes and encode more than 40 different isoforms (Wang and Coluccio, 2010). By contrast, the Drosophila genome contains two tropomyosin genes, tm1 and tm2. Genome annotation suggests that both genes are capable of producing multiple alternatively spliced isoforms. However, not much is known regarding the expression profile of the various tropomyosin isoforms in non-muscle tissues. A recent study by Goins and Mullins revealed that three tropomyosin isoforms are expressed in Drosophila S2 cells; Tm1A, Tm1J and Tm2A (Goins and Mullins, 2015).

The Tropomyosin isoform that interacts with Khc is Tm1C or Tm1I. These isoforms encode the same protein but differ in their untranslated regions. For simplicity, we refer to this Khc-interacting isoform as Tm1C. Although Kinesin has been shown to interact with a Myosin motor (Huang et al., 1999), this is the first report of a Tropomyosin that is capable of directly binding a microtubule motor. In comparison to canonical Tropomyosins, Tm1C has a unique domain organization. Most tropomyosins, including the high molecular weight isoforms detected in mammals, are composed of coiled-coil domains that enable binding along the sides of actin filaments (Gunning et al., 2015). Interestingly, the high molecular weight isoforms in mammalian cells are still less than 300 amino acids long. By contrast, Tm1C encodes a 441 amino acid protein. In addition to being much larger than classical Tropomyosins, Tm1C also contains a long N-terminal extension that is devoid of coiled-coil motifs (Fig. 1E).

How might Tm1C function in osk localization? One possibility is that Tm1C might be required for organization of the microtubule cytoskeleton. Thus, a defect in this process could indirectly result in osk localization. Our results suggest that this scenario is unlikely. The distribution of alpha-tubulin, a core component of the microtubule polymer, was the same in control and Tm1C null egg chambers (Supplemental Fig. 4E, F). In addition, no difference was detected in the distribution of microtubule minus-ends, and Kin:βgal, a reporter of microtubule plus-ends (Clark et al., 1994), remained correctly localized at the posterior pole in Tm1C nulls (Supplemental Fig. 4G-J). Lastly, cargoes such as bcd and grk mRNA, whose localization requires a correctly polarized cytoskeleton, were correctly sorted in egg chambers lacking Tm1C (Fig. 5 I, L, Supplemental Fig. 4A).

We propose a model whereby Tm1C directly or indirectly links Khc to the osk mRNP. This is supported by the observation that loss of either Tm1C or Khc produces identical osk delocalization phenotypes (Figs. 4 and 6). In both instances, osk mRNA localized around the oocyte cortex. In addition, we demonstrate that Tm1C binds to a small domain in the C-terminal tail of Khc (Fig. 2). Consistent with the adaptor model, Williams et al., have
shown that osk mRNPs are delocalized around the cortex in mutants expressing a truncated version of Khc that is lacking this domain (Williams et al., 2014).

A key prediction of this hypothesis is that in the absence of Tm1C, Khc no longer associates with the osk mRNP. Although we have been able to detect osk mRNA in Staufen immunoprecipitates, the same technique has proven unsuccessful in detecting osk in Khc immunoprecipitates (data not shown). Thus, although there is undisputed evidence that Khc transports osk mRNA (Zimyanin et al., 2008), there have been no published reports demonstrating a physical interaction between Khc and osk. A likely possibility is that motor-cargo complexes are labile, and therefore not retained during biochemical purification. Consistent with this notion, although Khc is thought to transport Dynein to the posterior pole (Brendza et al., 2002; Duncan and Warrior, 2002; Januschke et al., 2002), we failed to detect any peptides from Dynein in Khc immunoprecipitates (Table 1 and data not shown). Given this limitation in detecting the Khc-osk interaction in wild-type egg chambers, it has not been possible to prove a lack of association in tm1_C nulls.

Several years back, Erdélyi et al. and Tetzlaff et al., demonstrated a requirement for cytoplasmic tropomyosin in the localization of osk mRNA (Erdélyi et al., 1995; Tetzlaff et al., 1996). In these papers, the gene was referred to as tmII (Erdélyi et al., 1995; Tetzlaff et al., 1996). However, current genome annotation suggest that this P-element resides within the tm1 gene. Furthermore, the position of the P-element transposons used in these publications suggests that the isoform most likely affected in these mutants is Tm1A. The mechanism by which loss or reduction of Tm1A could contribute to osk mRNA delocalization is unknown. However, it is unlikely to function via Kinesin. Unlike Tm1C, Khc does not appear to associate with Tm1A (Fig. 1C). In addition, osk accumulates at the anterior margin of these previous described tropomyosin mutants and not along the oocyte cortex (Erdélyi et al., 1995; Zimyanin et al., 2008). If indeed Tm1A also participates in the localization of osk mRNA, further studies will be required to reveal its mechanism of action.

Although tm1_delC adult females can be obtained, the mutant is semi-lethal. Approximately 20% of homozygotes die during pupal stages. By contrast, complete loss of osk is not associated with lethality. This raises the possibility that Tm1C has additional functions, and loss of these functions, contributes to partial lethality. Consistent with this notion, Tm1C could be detected in whole males (Supplemental Fig. 4C). In addition, RNA-seq results suggest that unlike osk, this unique isoform of Tropomyosin is expressed at several developmental stages (Supplemental Fig. 4D) (Graveley et al., 2011). Future studies will seek to define whether Tm1C functions in concert with Khc to transport additional cargoes or whether Tm1C has roles that are independent of Kinesin-1.

While our manuscript was in revision, Cho et al., independently identified a role for Tm1C in border cell migration (Cho et al., 2016). Furthermore, these authors demonstrated that Tm1C shares some properties with intermediate filaments (Cho et al., 2016). Under in vitro conditions, Tm1C was able to form filamentous structures. In addition, when ovaries were incubated with a PIPES pH 6.8 buffer, mCherry-Tm1C formed filaments in vivo that colocalized with microtubules (Cho et al., 2016). We obtained similar results using our GFP-Tm1C strain (Supplemental Fig. 2G). Interestingly, a similar localization pattern was also observed for Khc using these same fixation conditions (Supplemental Fig. 2H, I). However, Khc maintained this filament/microtubule
localization pattern in tm1_delC nulls (Supplemental Fig. 4K, L). Thus, the role of Tm1C in the osk localization pathway does not involve recruitment of Khc to microtubules. As stated previously, we favor a model whereby Tm1C links Khc to osk mRNPs, thus facilitating posterior transport of this mRNA.
Materials and Methods

Fly stocks
The following stocks were used: Oregon-R-C (used as wild-type; Bloomington stock center; #5); GFP-Stau (Zimyanin et al., 2008); eb1 shRNA (Bloomington stock center; #36680, donor TRiP); egl shRNA-1 (Bloomington stock center; #43550, donor TRiP); khc shRNA (Bloomington stock center; #35409, donor TRiP), and Kin:βgal (Clark et al., 1994). shRNA expression was driven using P[w[+mC]=matalpha4-GAL-VP16]V37 (Bloomington stock center; #7063, donor Andrea Brand).

GFP-Tm1C was expressed by cloning the Tm1C coding sequence into a vasa-GFP expression vector (Sano et al., 2002). The Tm1C coding sequence with silent-mutations at the shRNA site was synthesized by Genewiz. tm1 shRNA flies were generated by cloning the following sequences into the NheI and EcoR1 sites of the Valium 22 vector (Ni et al., 2011):

- tm1 shRNA-1: 5′-GCCGACGACGATGACAACCAA-3′

- tm1 shRNA-2: 5′-AAGGTCAGAGAAATCGGACAA-3′

Transgenic flies containing these constructs were generated by BestGene Inc (inserted at the attP40 site on chromosome 2).

tm1_delC nulls were created by injecting a mix containing two guide RNA plasmids along with a donor vector for homology directed repair (Gratz et al., 2014). Complete details regarding the cloning strategy will be provided upon request.

Antibodies and imaging reagents
Unless specifically stated, the indicated dilutions are for immunofluorescence: mouse anti-Dhc (Developmental studies hybridoma bank; 1:150; donor J. Scholey); rabbit anti-Khc (Cytoskeleton, Inc., 1:150, western 1:1500); chicken anti-Oskar (1: 50); FITC conjugated mouse anti-alpha tubulin (Sigma; 1: 100); mouse anti-β galactosidase (Promega; 1:1000); mouse anti-gamma-tubulin (Sigma; 1:100); rat anti-GFP (Nacalai USA, Inc.; 1:600); mouse anti-LaminDmO (Developmental studies hybridoma bank; clone ADL84.12; 1:200; donor P.A. Fisher); mouse anti-GFP (Clontech, western 1:4000); rat anti-RFP (Chromotek, western 1:1000); rabbit anti-Vas (1:1000 from P. Lasko); Tm1C (western 1:1500); goat anti-rabbit Alexa 594 and 488 (Life technologies, 1:400 and 1:200 respectively); goat anti-mouse Alexa 594 and 488 (Life technologies, 1:400 and 1:200); goat anti-chicken 594 (Life technologies, 1:400). Tm1C antibody was generated by injecting the following peptides into rabbits:

DKSEKSDRRKKSSGKERSKRSNP
KEARFLAEEDKKYDEVQLK

The injection and purification of peptide-specific antibodies was performed by Pacific immunology (Ramona, CA). TRITC conjugated phalloidin (Sigma Aldrich) was used to visualize F-actin and ToPro3 (Life Technologies) was used to visualize DNA.
DNA constructs
For binding studies in S2 cells, cDNA for tm1C and klc was cloned into the pAGW gateway vector; khc constructs were cloned into the pARW vector (obtained from the Drosophila Genomics Resource Center). Internal deletion in khc were made using the Q5 site-directed mutagenesis kit (NEB, Ipswitch MA). Transfections were performed using Effectene (Qiagen). For the direct binding experiment, tm1C was cloned into pDEST15 and the khc cDNA was cloned into the pSP64 vector (Promega). tm1C guide RNAs were cloned into the pU6-3 Bbsi plasmid and tm1C homology arms were cloned into the pHd-DsRed-attP plasmid from the lab of Kate O’Connor-Giles. For purification of GFP and Khc-GFP from ovarian lysates, the coding sequence of either GFP or Khc-GFP was cloned into the pUASp-attB-K10 vector (Koch et al., 2009).

Protein-protein interaction
In order to purify Khc interacting partners, ovaries were dissected from flies expressing either GFP or Khc-GFP. The ovaries were homogenized into lysis buffer (50mM Tris pH 7.5, 50mM NaCl, 0.2mM EDTA, 0.05% NP40 and Halt protease inhibitor cocktail, Pierce). The lysates were cleared by centrifugation at 10,000g at 4°C for 5min. 1000ug of either GFP or Khc-GFP expressing lysate was added to GFP-trap beads (Chromotek). The binding was performed at 4°C for 1hour. Next, the beads were washed twice with NP40 containing wash buffer (50mM Tris pH 7.5, 200mM NaCl, 0.2mM EDTA, 0.05% NP40). Subsequently, the beads were washed twice with the same buffer but lacking NP40. Next, the co-precipitating proteins were eluted using a 0.2M Glycine pH 2.5 solution. After elution, the pH was neutralized using Tris pH 8.0. The samples were digested with trypsin and analyzed by tandem mass spectrometry. The trypsin digestion and mass spectrometry analysis was performed at the Mass Spectrometry and Proteomics Resource Laboratory at Harvard University.

For detecting protein-protein interaction using S2 cells, lysates were prepared using the indicated transfected cells. The cells were lysed using RIPA buffer (50mM Tris-Cl pH 7.5, 150mM NaCl, 1% NP40, 1mM EDTA). The lysates were cleared by centrifugation as described above and added to GFP-trap beads (Chromotek). After binding and wash steps, the co-precipitating proteins were eluted in Laemmli buffer, run on a gel, and analyzed by western blotting.

For the direct binding studies, GST and GST-Tm1C was expressed in BL21 DE3 pLysS cells (Life Technologies). The proteins were induced using 0.5mM IPTG for 6 hours at room temperature. Lysates were prepared using a French Press. The induced proteins were purified by incubation with Glutathione sepharose beads (Pierce). GelCode Blue (Pierce) was used to visualize these proteins in a gel. The RFP-tagged Khc construct was expressed using the TnT SP6 Coupled Wheat Germ Extract System (Promega).

Immunofluorescence and in situ hybridization
Ovaries were processed for immunofluorescence as previously described (Sanghavi et al., 2016). In situ hybridization to detect bcd, grk, nos and cycB mRNA was performed using a published procedure (Sanghavi et al., 2013). smFISH was used for detection of osk. Quasar570 conjugated Stellaris DNA oligonucleotide probes against osk and
gapdh1/2 were obtained from LGC Biosearch technologies (Petaluma, Ca). Ovaries were
fixed in 4% formaldehyde (diluted in PBS) for 20minutes. Subsequently, the ovaries were
washed with several changes of PBS. Next, methanol was added and the ovaries were
stored at -20°C for 1hour. The ovaries were then washed with a 7:3 methanol:PBST (PBS
plus 0.1% triton X-100) solution for 10 minutes at room temperature. Next, 3:7
methanol:PBST was used. The ovaries were then washed 4x with PBST. PBST was
removed and the samples were incubated in prehybridization buffer (4xSSC, 35%
deionized formamide, 0.1% Tween-20) for 10minutes. The prehybridization solution was
removed and the probe diluted to 100nM in hybridization buffer (10% dextran sulfate,
0.1mg/ml salmon sperm ssDNA, 100 µl vanadyl ribonucleoside (NEB biolabs, Ipswitch
MA), 20ug/ml RNAse-free BSA, 4x SSC, 0.1% Tween-20, 35% deionized formamide)
was added. The ovaries were incubated with probe overnight at 37°C. The next day, the
probe was removed and the ovaries were washed twice in prehybridization buffer for
30minutes each. The samples were then stained with DAPI, washed in PBST, and
mounted onto slides using Aqua Poly mount (Polysciences, Inc, Warrington PA). In order
to detect filaments of GFP-Tm1C and Khc, ovaries expressing the transgene were processed as
previously described (Cho et al., 2016).

Microscopy
Images were captured on a Zeiss LSM 780 upright confocal microscope and were
prepared for presentation using Fiji, Adobe Photoshop and Adobe Illustrator.

Quantification
Localization phenotypes were quantified by scoring oocytes of the indicated genotype
from three independent experiments. The level of GFP-Tm1 in stage 10 egg chambers
(Fig.3K) was quantified using the Zen software (Zeiss). The level of fluorescence intensity
in a 50 micron-square area in nurse cells of control and Khc depleted egg chambers was
determined. The quantification was done on 25 different egg chambers using a single
confocal slice. An unpaired t-test was performed using standard deviation, mean and n
value.
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Figure 1: Tm1C is an interacting partner of Khc.

(A) Schematic of the Tm1 locus. Untranslated regions are in grey and coding regions are depicted in orange.

(B) Co-precipitation experiment in S2 cells between RFP-Khc and GFP (lanes 1, 3) or GFP-Tm1C (lanes 2, 4). The Khc construct did not contain the motor domain. The immunoprecipitation was performed using GFP-trap beads. The co-precipitating proteins and total fraction were analyzed by blotting using the indicated antibodies.

(C) A co-precipitation experiment between RFP-Khc and GFP-Tm1C (lanes 1, 5), Tm1J (lanes 2, 6), Tm2A (lanes 3, 7) or Tm1A (lanes 4, 8). The immunoprecipitation was performed as in panel B and the co-precipitating proteins were detected by blotting using the indicated antibodies. The arrow indicates full-length GFP-Tm1C and the arrow-head denotes full-length GFP-TmJ, Tm2A and Tm1A. Over-expression of GFP tagged Tropomyosins in S2 cells often resulted in smaller, faster migrating bands (indicated by asterisks in this panel). We assume these bands represent breakdown products resulting from protein turnover.

(D) A direct binding experiment using recombinant GST or GST-Tm1C produced in bacteria and RFP-Khc produced using in vitro transcription and translation. The binding reaction was run on a gel and analyzed by blotting using an anti-RFP antibody (top panel). The bottom panel is a GelCode blue stained gel showing the amount of GST and GST-Tm1C used in the binding reaction.

(E) The domain structure of Tm1A, Tm1J and Tm1C proteins are shown at the top of the figure. Numbers correspond to the amino acid positions for the various domains. The bottom part of the figure shows a co-precipitation experiment using RFP-Khc and either GFP (lanes 1, 5), full length GFP-Tm1C (lanes 2, 6), an N-terminal GFP-Tm1C construct (lanes 3, 7) or a C-terminal GFP-Tm1C construct (lanes 4, 8). Immunoprecipitation was performed as in panel B. The arrow indicates full-length GFP-Tm1C, the arrow-head corresponds to N-terminally truncated GFP-Tm1C and the asterisk denotes C-terminally truncated GFP-Tm1C.
Figure 2: Tm1C binds to a C-terminal domain within Khc.

(A) Domain structure of Khc. The following features are indicated: motor domain, coiled-coil domains required for dimerization of Khc (shown in green), Klc binding site, a region important for cargo binding in N. crassa, ATP-independent microtubules binding region, and an auto-inhibitory domain. Also shown is an alignment of amino acids 914 to 936 of Khc with Kif5b, the human homolog of Khc.

(B) A co-precipitation experiment using various RFP-Khc truncation constructs with full-length GFP-Tm1C. The residues included in the truncations are indicated. Lysates were
prepared and the GFP tagged proteins were immunoprecipitated. The co-precipitating proteins and the total fraction were analyzed by blotting using the indicated antibodies.

(C) The same truncation constructs used in the previous panel were co-transfected with full-length GFP-Klc. The experiment was performed as in panel B.

(D) A co-precipitation experiment using a small RFP-Khc construct and either GFP-Klc (lane 1) or Tm1C (lane 2). The immunoprecipitation was performed as described and analyzed using the indicated antibodies.

(E) A co-precipitation experiment using GFP-Tm1C and either full-length RFP-Khc or RFP-Khc containing the indicated deletions. The co-precipitation was performed as described and analyzed by blotting using the indicated antibodies.
Figure 3: Localization of GFP-Tm1C in the female germline.

(A-D) Localization of GFP-Tm1C at various stages of egg chamber maturation. DAPI staining of nuclei is indicated in red. The arrow indicates enrichment of GFP-Tm1C at the anterior of stage 7 oocytes and the arrow-head indicates posterior enrichment.

(E-F) Ovaries from the same strain were fixed and processed using an antibody against GFP (green, E, F) and Khc (red, E', F'). A merged image is also shown (E'', F'').

(G-H) Egg chambers from the same strain were processed using an antibody against GFP and were counter-stained with TRITC-phalloidin to visualize F-actin. GFP-Tm1 signal is depicted in G. Actin signal is depicted in G' and merged signal is shown in G'', H' and H''. H'' is a magnified view of the posterior pole.

(I-J) Egg chambers from strains expressing GFP-Tm1 and either a control shRNA against eb1 (I) or an shRNA against khc (J) were fixed and processed for immunofluorescence using an antibody against GFP. The samples were also counter-stained with TRITC-conjugated phalloidin. J', J'', and J''' represent individual and merged images from the boxed region in J.

(K) The intensity of GFP-Tm1 signal in nurse cells of control (eb1 shRNA) or khc depleted egg chambers was quantified. The error bars represent standard deviation. *** p = less than 0.0001, unpaired t test.

Scale bars: 50 microns (A-D, G, H, I, J); 25 microns (E, H'); 5 microns (J', J'', J''').
Figure 4: Depletion of Tm1C results in osk mRNA delocalization.

(A) Ovarian lysates from flies expressing a control shRNA (lane 1), tm1 shRNA-1 (lane 2), or tm1 shRNA-2 (lane 3) were analyzed by blotting using an antibody against Tm1C (top panel). This antibody also detects Tm1J. The blot was subsequently stripped and probed with an antibody against gamma tubulin (bottom panel).

(B-D) Ovaries from these strains were dissected and processed for single molecule in situ hybridization (smFISH) using oligonucleotide probes against osk mRNA. The osk signal (white) is super-imposed over a bright-field image.
The localization pattern of osk mRNA was quantified in the following strains: control shRNA, \(tm1\) shRNA-1, \(tm1\) shRNA-2, and a strain co-expressing \(tm1\) shRNA-2 and the GFP-Tm1C transgene. The number of egg chambers scored is indicated.

Ovaries from females expressing a control shRNA were processed for smFISH using probes against osk (red). The samples were counterstained with DAPI (cyan). \(F'\) and \(F''\) are enlarged images of boxes in \(F\). A stage 14 egg chamber is shown in \(G\), the posterior of which is enlarged in \(G'\).

Ovaries from females expressing \(tm1\) shRNA-2 were processed as above. \(H', H''\) and \(H'''\) are enlarged images of boxes in \(H\). Panel \(I\) is a stage 14 egg chamber and \(I'\) shows an enlargement of the posterior pole.

Similar layout to the above panels using flies expressing an shRNA against \(khc\).

Ovaries from females expressing \(egl\) shRNA-1 were processed as above. \(L', L''\) and \(L'''\) are enlarged images of boxes in \(L\). The dashed line in \(F', H', J'\) and \(L''\) indicate the border between nurse cells and the oocyte. Scale bars: 50 microns (B-D, F-L); 5 microns (\(F', F'', G', H', H''', I', J', J'', K', L', L''\), \(L''')\).
Figure 5: Several Khc-dependent processes are unaffected upon Tm1C depletion. (A-C) Ovaries were fixed from flies expressing either a control shRNA (A), khc shRNA (B), or tm1 shRNA-2 (C) and were counter-stained with TRITC-phalloidin to visualize F-actin.

(D-F) Ovaries were dissected from the same strains used in the preceding panels. The egg chambers were fixed and processed using an antibody against Lamin DmO (green). Arrows indicate the oocyte nucleus.

(G-I) Ovaries from flies expressing a control shRNA (G), khc shRNA (H), or tm1 shRNA-2 (I) were processed for in situ hybridization using probes against grk mRNA (green). The egg chambers were counter-stained with ToPro3 (red).
(J-L) The same strains were fixed and processed for situ hybridization using probes against bcd mRNA (green). The egg chambers were counter-stained with ToPro3 (red).

(M-N) Embryos were collected and fixed from control shRNA (M) or tm1 shRNA-2 (N) expressing mother. The embryos were processed for in situ hybridization using probes against nanos mRNA (nos, green). M' and N' represent blastoderm stage egg chambers from the indicated strains. The posterior region of these embryos is shown.

(O-P) Embryos from these same strain were fixed and processed for in situ hybridization using probes against cyclin B mRNA (cycB, green). As with the above panel, O' and P' represent blastoderm stage egg chambers from the indicated strains. Quantification of phenotypes and the number of egg chambers or embryos scored are indicated. Scale bars: 50 microns (A-P); 25 microns (M', N', O', P').
Figure 6: Generation of a tm1C null.

(A) Schematic showing the region deleted in tm1C nulls. We refer to this strain as \textit{tm1\_delC}. Grey boxes represent untranslated regions and orange boxes indicate coding regions. Sequences of the guide RNAs used to generate this deletion are also indicated.

(B) Ovarian lysates were prepared from wild-type (lane 1) or \textit{tm1\_delC} nulls (lane 2). The lysates were analyzed by blotting using the Tm1C antibody. The blot was subsequently stripped and probed with an anti-Khc antibody (bottom panel).

(C-D) Ovaries from \textit{tm1\_delC} nulls were processed for smFISH using probes against osk (red) and were counter-stained with DAPI (cyan). D', D'' and D''' are enlarged images of boxes in D.

(E) Ovaries from tm1\_delC nulls expressing the GFP-Tm1C transgene were processed for smFISH using probes against osk (red) and were counter-stained with DAPI (cyan). The arrow indicates localized osk mRNA.

(F-G) Ovaries from wild-type (F) or \textit{tm1\_delC} nulls (G) were fixed and processed for immunofluorescence using an antibody against Vasa. The arrow indicates localization of Vasa to the pole plasm in wild-type egg chambers.
(H-I) Ovaries from tm1_delC nulls were processed for immunofluorescence using antibodies against Khc (H) or Dhc (I). Arrows indicated posterior-localized Khc and Dhc in tm1C nulls.
Scale bars: 50 microns (C-I); 5 microns (D', D'', D''').
The table lists proteins that were identified as co-precipitating with either GFP or Khc-GFP. The number of peptides obtained for these proteins as well as their respective spectral count is indicated. Proteins that were found at relatively equivalent levels in both pellets were considered proteomic contaminants and were therefore excluded from this list. Most of these contaminants corresponded to ribosomal proteins. Proteins that were represented by fewer than two peptides were also excluded.
Bibliography


Supplemental fig 1: Tm1 locus, mass spectrometry results and interaction with Khc.

(A) Schematic of the tm1 genomic locus adapted from FlyBase.

(B) Tm1 peptides obtained in the Khc-GFP pellet and the Tm1 isoforms which contain the indicated peptides.

(C) Ovarian lysates were prepared from flies expressing either GFP (lane 1) or Khc-GFP (lane 2). The lysate was added to GFP-trap beads. The immunoprecipitating proteins were examined using the indicated antibodies.

(D) A complementary experiment using flies expressing either GFP (lane 1) or GFP-Tm1C (lane 2). As with panel A, the lysate was added to GFP-trap beads and the co-precipitating proteins were analyzed using the indicated antibodies.
Supplemental fig 2: Localization of GFP-Tm1C.

(A) GFP-Tm1C (A) co-localizes with the nuage marker, Aubergine (Aub, red, A'). A' represents a merged image.

(B) A strain expressing unfused GFP in the germline is shown. DAPI (cyan) and GFP signals are overlaid in B'.

(C-F) GFP-Tm1C localization in control (C, C', E, E') or LatA treated (D, D', F, F') egg chambers. The green signal indicates the localization of GFP-Tm1C and the red signal indicates the presence of F-actin.

(G-I) Ovaries from flies expressing GFP-Tm1C were incubated with a PIPES pH 6.8 buffer for 1 hours prior to fixation in order to visualize filaments (Cho et al., 2016). The samples were then processed using antibodies against GFP (G) or Khc (H). A merged image is shown in I. G', G'', H', H'', I' and I'' are enlarged images of the boxes shown in the main figure.

Scale bars: 50 microns (A-F); 25 microns (G, H, I); 5 microns (G', G'', H', H'', I' and I').
**Supplemental fig 3: Depletion phenotypes and smFISH controls.**

(A) Khc levels and localization in egg chambers expressing a control shRNA (A) or shRNA targeting khc (A').

(B) Lysates from flies expressing a control shRNA (lane 1) or shRNA against khc (lanes 2 and 3) were run on a gel and examined by western blotting using the indicated antibodies.

(C) smFISH performed on wild-type egg chambers using probes against gapdh 1 and 2 (red). The samples were counterstained with DAPI (cyan). C' and C'' are enlarged images of the boxes shown in C.

(D-F) Localization of GFP-Stau in egg chambers expressing a control shRNA (D), tm1 shRNA-1 (E) or tm1 shRNA-2 (F).

(G-H) Localization of Osk (G, H) and Vas (G', H') in control egg chambers (G) or egg chambers expressing tm1 shRNA-2 (H). Merged images are shown in G'' and H''.

(I-K) Localization of GFP-Tm1C (I, I'), osk mRNA (J) or Vasa (K) in egg chambers co-expressing tm1 shRNA-2 and GFP-tm1c. Scale bars: 5 microns (C', C''); 50 microns for the rest.
Supplemental fig 4: Tm1C null phenotype, expression pattern and polarity.

(A) Ovaries from tm1_delC nulls were fixed and processed for in situ hybridization using probes against grk mRNA (green). The arrow indicates localized grk mRNA in tm1_delC oocytes.

(B) Localization of the oocyte nucleus shown using Lamin DmO staining (green). F-actin is shown using TRITC conjugated phalloidin (red).

(C) Lysates from wild-type males (lanes 1, 3) or tm1_delC males (lanes 2, 4) were examined by western blotting using the Tm1C antibody.

(D) RNA-seq data from Graveley et al (Graveley et al., 2011). Image adapted from FlyBase.

(E-F) Localization of alpha-tubulin in wild-type (E) or tm1_delC nulls (F).

(G-H) Localization of gamma-tubulin in wild-type (G) or tm1_delC nulls (H).

(I-J) Localization of Kin:βgal (red) in control (I) or tm1_delC null (J) egg chambers. The egg chambers were counterstained with DAPI (cyan). Arrows indicate posterior localized Kin:βgal.

(K-L) Ovaries from wild-type (K) or tm1_delC nulls (L) were incubated with a PIPES pH 6.8 buffer for 1 hour prior to fixation in order to visualize filaments. The samples were then processed using an antibody against Khc. Khc localized to filaments in the presence or absence of Tm1C. Scale bars: 50 microns.