Molecular basis of mRNA transport by a kinesin-1–atypical tropomyosin complex

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Kinesin-1 carries cargos including proteins, RNAs, vesicles, and pathogens over long distances within cells. The mechanochemical cycle of kinesins is well described, but how they establish cargo specificity is not fully understood. Transport of oskar mRNA to the posterior pole of the Drosophila oocyte is mediated by Drosophila kinesin-1, also called kinesin heavy chain (Khc), and a putative cargo adaptor, the atypical tropomyosin, aTm1. How the proteins cooperate in mRNA transport is unknown. Here, we present the high-resolution crystal structure of a Khc–aTm1 complex. The proteins form a tripartite coiled coil comprising two in-register Khc chains and one aTm1 chain, in antiparallel orientation. We show that aTm1 binds to an evolutionarily conserved cargo binding site on Khc, and mutational analysis confirms the importance of this interaction for mRNA transport in vivo. Furthermore, we demonstrate that Khc binds RNA directly and that it does so via its alternative cargo binding domain, which forms a positively charged joint surface with aTm1, as well as through its adjacent auxiliary microtubule binding domain. Finally, we show that aTm1 plays a stabilizing role in the interaction of Khc with RNA, which distinguishes aTm1 from classical motor adaptors.

Keywords: kinesin; kinesin adaptor; kinesin–atypical tropomyosin complex; mRNA transport; oskar mRNA

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Intracellular transport is an essential cellular function that is ensured by a vast cytoskeletal network and three major classes of motor proteins, namely kinesins, dyneins, and myosins [Hirokawa et al. 2009; de Lanerolle 2012; Olenick and Holzbaur 2019]. Whereas myosins are responsible for short-range transport along randomly oriented actin filaments, kinesins and dyneins provide long-range directional transport on a polarized microtubule network [Titus 2018]. Kinesin-1, a microtubule plus-end-directed motor, was the first kinesin identified and is well characterized [Vale et al. 1985; Hirokawa et al. 2009]. It transports diverse cargos including proteins, organelles, mRNA nucleoprotein complexes [mRNPs], and viruses [Chudinova and Nadezhdina 2018; Garcin and Straube 2019; Banerjee et al. 2020] and is essential for numerous processes such as endoplasmic reticulum (ER)-Golgi transport, mitochondrial distribution in axons and dendrites, cell migration, and embryonic axis formation [Hirokawa et al. 2009].

Kinesin-1 harbors an N-terminal motor domain, followed by a long coiled-coil stalk that mediates homodimerization, and a flexible tail with regulatory function [Fig. 1A; Seeger and Rice 2013]. The two motor domains are powered by ATP hydrolysis, which elicits conformational changes that allow the kinesin molecule to move processively along microtubules [Qin et al. 2020]. The kinesin-1 stalk associates with cargos either directly or via adaptor proteins that bind to the C-terminal region of the domain [Cross and Dodding 2019]. Although a large portion of kinesin-1 transport is mediated by the adaptor kinesin light chain (Klc), the transport of mRNA cargos such as mRNP granules in dendrites or oskar in the Drosophila oocyte appears to be independent of Klc [Diefenbach et al. 1998; Kanai et al. 2004; Loiseau et al. 2010; Cross and Dodding 2019].

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In *Drosophila*, localization of *oskar* mRNA to the posterior pole of the developing oocyte drives abdominal patterning and germline formation in the embryo [Ephrussi and Lehmann 1992]. The directional transport of RNA is mediated by both the dynein and kinesin transport machineries (Clark et al. 2007; Zimyanin et al. 2008). In the germline syncytium, the oocyte is transcriptionally quiescent and relies on mRNAs provided by 15 interconnected nurse cells for its growth and development (Roth and Lynch 2009). Upon transcription in the nurse cells, *oskar* mRNA is transported into the oocyte on the microtubule cytoskeleton by the minus end-directed dynein motor complex (Clark et al. 2007). Within the oocyte, the microtubule plus end-directed motor kinesin-1 or kinesin heavy chain (Khc) in *Drosophila* [Lawrence et al. 2004] transports *oskar* to the posterior pole where the mRNA is localized [for review, see Trcek and Lehmann 2019].

Moreover, kinesin-mediated *oskar* transport requires a number of cis and trans factors. One of these factors is a unique isoform of the actin binding protein tropomyosin 1, known as Tm1, which we refer to here as atypical tropomyosin 1 (Loiseau et al. 2010; Veeranan-Karmegam et al. 2016; Gáspár et al. 2017a). In the absence of atypical tropomyosin 1, *oskar* mRNA fails to localize to the posterior pole of the oocyte, as kinesin recruitment to *oskar* mRNA requires atTm1 function [Erdélyi et al. 1995; Gáspár et al. 2017a].
2017a). Furthermore, the fact that tethering of the Khc motor domain to oskar restores posterior localization of the mRNA in a Tm1 protein-null background suggested that aTm1 is an essential adaptor for Khc-mediated oskar transport (Gáspár et al. 2017a). Here, we show that aTm1 homodimers adopt an unusual antiparallel coiled-coil conformation. Moreover, aTm1 binds to an alternative conserved cargo binding region in Khc, forming a tripartite coiled-coil complex with a positively charged surface. Finally, we show in vivo that the function of aTm1 is ensured by its Khc interacting domain and that this atypical Tm1 functions in stabilizing the interaction of Khc with RNA rather than as a classical adaptor.

Results

aTm1 binds to the alternative cargo binding site of Khc

It was previously reported that aTm1 is necessary for the recruitment of Khc to oskar mRNA and that the two proteins physically interact (Veeranan-Karmegam et al. 2016; Gáspár et al. 2017a). To elucidate the molecular basis of the aTm1-Khc interaction, we first performed a yeast two-hybrid analysis, which confirmed that aTm1 (Tm1-FL) binds both full-length Khc (Khc-FL) and Khc848-975 [Fig. 1B]. Because the interaction of Khc and aTm1 was formerly tested by coimmunoprecipitation (IP) experiments from S2 cells or Drosophila ovarian lysates, neither of which is performed in a nucleic acid-free environment (Veeranan-Karmegam et al. 2016; Gáspár et al. 2017a), we also probed the interaction by pull-down of recombinant Khc and aTm1 proteins in the presence of DNase and RNAase [Fig. 1C, inputs in Supplemental Fig. S1A]. Binding of aTm1 to Khc was preserved under these conditions [Fig. 1C, right panel, lanes 5,6], indicating the specific and direct interaction of aTm1 with Khc.

To further narrow down the aTm1 and Khc interacting regions, we performed GST pull-down assays on shorter fragments of the proteins (Fig. 1D; Supplemental Fig. S1B,C). This revealed that aTm1247–335 binds Khc848-941 [Fig. 1D, panel 4], which comprises the alternative cargo binding region and the ATP-independent microtubule binding region of Khc [Fig. 1D; Seiler et al. 2000; Williams et al. 2014]. Veeranan-Karmegam et al. (2016) have previously suggested that aTm1 interacts with residues 914–936 of Khc. However, the fact that Khc902–941 did not interact with aTm1 in our assays [Fig. 1D, panel 5] indicates that aTm1247–335 binds to the conserved alternative cargo binding site of Khc [residues 848–902] [Supplemental Fig. S1D].

The alternative cargo binding region of Khc is essential for oskar mRNA localization

The alternative cargo binding region in Khc was originally identified in Neospora crassa kinesins (Nkin), where deletion of this site abolished the ability of the protein to rescue the Nkin null mutant phenotype (Seiler et al. 2000). In Drosophila, deletion of the entire C terminus of Khc, which includes the alternative cargo binding region and the regulatory tail, also led to an oskar mRNA localization defect among others (Williams et al. 2014). To investigate the relevance of this cargo binding region for oskar mRNA transport, we expressed RNAi-resistant Khc-FL-mKate2 and Khc855–911A-mKate2 [wherein the cargo binding domain is deleted] fusion proteins in a khc-RNAi background [Fig. 2A; Supplemental Fig. S2]. Khc855–911A localized to the posterior pole of the oocyte [Fig. 2B, upper right panel], indicating that Khc motor function and polarity of the microtubule cytoskeleton were preserved. However, oskar mRNA failed to localize at the posterior pole [Fig. 2B, bottom right panel] and the eggs produced failed to hatch [Fig. 2C], demonstrating that the alternative cargo binding region of Drosophila Khc, and likely its interaction with aTm1, play a major role in oskar mRNA transport.
The Khc interaction domain of aTm1 is essential for oskar localization

The region of aTm1 (residues 247–335) that interacts with Khc consists of a coiled coil that is also present in the classical Tm1-A isoform (residues 282–335), preceded by a unique N-terminal sequence predicted to be at least partially helical (Fig. 1D). To understand the functional significance of the aTm1–Khc interaction, we generated GFP-tagged aTm1 truncations in which the Khc interaction site was either present or absent and tested them in vivo. We expressed the constructs in Tm1eg9 mutant flies, in which aTm1 is not present and oskar mRNA fails to localize at the posterior of the oocyte (Supplemental Fig. S3A–C, first panel; Erdélyi et al. 1995; Gáspár et al. 2017a).

Expression of transgenic full-length aTm1 (aTm1FL) and aTm11–334 restored oskar localization to the posterior pole of Tm1eg9 oocytes (Fig. 3A–C), suggesting that the aTm1 C-terminal region is nonessential for this function. Expression of the minimal Khc interaction region of aTm1 (aTm1247–334) also promoted oskar localization in Tm1eg9 oocytes. Although the RNA was not as concentrated as expected at the posterior pole as upon aTm1FL or aTm11–334 expression (Fig. 3B,C, analysis was performed as in Gaspar et al. 2014), the female progeny developed ovaries and were fertile. Neither aTm11–247 nor aTm1335–441 could restore oskar localization in Tm1eg9 oocytes (Supplemental Fig. S3C). These data show that the core domain of aTm1 (residues 247–334) identified as the Khc binding domain is essential for oskar mRNA localization.

Interestingly, despite supporting oskar mRNA localization, GFP-aTm1247–334, which lacks a major portion of the unique N-terminal region of aTm1, does not accumulate at the posterior pole of the oocyte, in contrast to aTm1FL or aTm11–334. This suggests that the aTm1 N-terminal domain interacts with components of the pole plasm or with oskar mRNA itself, resulting in aTm1 accumulation at the oocyte posterior.

aTm1 coiled-coil dimers adopt an antiparallel configuration

To gain insight into the molecular basis of aTm1–Khc interaction, we used crystallography to determine the structure of aTm1 alone and in complex with Khc. The classical actin-binding tropomyosins (Tm1-PG and Tm1-PA in Drosophila) are highly conserved in eukaryotes (Fig. 4A), where they form long parallel coiled coils that polymerize in a head-to-tail fashion along the actin filaments (Parry and Squire 1973). Residues 81–165 of Tm1-PG are present in aTm1 whose coiled-coil region is further extended by 20 unique residues at the N terminus (aTm1262–366) (Fig. 4A), which we included in our constructs.

We performed crystallization trials with different aTm1 truncations (Fig. 4A) and obtained crystals for Fig. 3. aTm1247–334 rescues oskar mRNA localization in Tm1eg9/Tm1eg9 mutant. [A] Confocal images of Tm1eg9/Tm1eg9 egg chambers expressing GFP-tagged aTm1FL, aTm11–334, and aTm1247–334 variants. oskar mRNA was visualized by smFISH. Scale bar, 25 µm. [B] Mean oskar distribution (green) in stage 9 oocytes from the flies in A. n indicates the number of oocytes analyzed. [C] Position of oskar mRNA center of mass relative to the geometric center of the oocyte represented in box plots with minimum and maximum whiskers. The analysis was performed as in Gaspar et al. [2014].
aTm1<sub>262–363</sub> and aTm1<sub>270–334</sub> constructs that diffracted to a resolution of 2.45 Å and 2.3 Å, respectively [Fig. 4B]. The structures were solved by ab initio molecular replacement with Ambler and were refined to an \( R \) work/\( R \) free of 0.25/0.29 and of 0.23/0.27 for aTm1<sub>262–363</sub> and aTm1<sub>270–334</sub>, respectively (see Supplemental Table S1 for structural statistics; Bibby et al. 2012). Residues 261–358 were fitted into the density of the aTm1<sub>262–363</sub> crystal structure, whereas residues 277–319 in the first coil and residues 273–330 in the second coil were resolved in aTm1<sub>270–334</sub>. Both truncations crystallized as antiparallel coiled coils with an average backbone RMSD of 1 Å between residues 278 and 328 [Fig. 4B]. A helical wheel diagram of aTm1<sub>262–363</sub> as generated by DrawCoil 1.0 [https://grigoryanlab.org/drawcoil]. The dashed lines connecting Arg292 and Asp299 indicate salt bridges between these residues.

aTm1–Khc interaction domains form a triple coiled coil of two kinesin chains and one aTm1 chain

We next reconstituted, biophysically characterized, and crystallized the minimal aTm1–Khc complex. To do so, the initially identified protein boundaries [Fig. 1D] were further refined to improve protein solubility and yields, after which individually purified aTm1<sub>252–334</sub> and Khc<sub>855–941</sub> were mixed and analyzed by gel filtration [Fig. 5A]. Upon addition of aTm1, the Khc peak shifted to a higher apparent molecular weight, indicating complex formation. Interestingly, the aTm1 peak fractions shifted to a later retention time [larger elution volume] indicative of compaction of the protein in the complex upon binding [Fig. 5A, middle gel] and lower gel [lanes 8–10]. Also, although we mixed the proteins in a 1:1 ratio before injection onto the column [Fig. 5A, right panel, lowest gel, lane 1], the ratio of the bands in the complex peak [Fig. 5A, right panel, lowest gel, lanes 8–12], as well as the presence of an additional aTm1 peak [Fig. 5A, right panel, lowest gel, lanes 3–7] indicate that Khc is in excess in the complex. To determine the affinity of the aTm1–Khc interaction, we performed surface plasmon resonance (SPR) experiments using an anti-GST antibody.
Figure 5. The aTm1–Khc complex forms a tripartite coiled coil. (A) Reconstitution of an αTm1–Khc complex, consisting of the minimal interaction regions of the two proteins (left schematic). Size exclusion chromatography (S200 resin) of Khc855–941, αTm1252–334, and a 1:1 mixture of both proteins. The UV trace of αTm1 alone could not be recorded because of the very low extinction coefficient of this truncation. Peak fractions were analyzed by gel electrophoresis in the bottom panel. (B) Binding of αTm1252–334 to Khc848–941 analyzed by surface plasmon resonance (SPR). GST-tagged Khc848–941 was used as a ligand, and increasing concentrations (indicated above the sensogram in nanomoles) of αTm1 were added as analyte. Ligand to analyte was fitted using a standard 1:1 kinetic model with a $K_d = 141 \pm 6 \text{nM}$. (C) Crystal structure of the αTm1252–334/Khc855–941 complex. Two different helical views (left panels) and the corresponding electrostatic views (right panels) are presented. The two Khc chains (aquamarine) are parallel and in register, whereas the single αTm1 chain (gold) is in an antiparallel conformation. The crystal structures consist of αTm1 (residues 259–324), KhcI (residues 855–923), and KhcII (residues 855–916). The bottom two left and right panels represent ribbon and electrostatic views of αTm1262–363 for comparison. (D) The triple coiled coil of the αTm1–Khc complex is stabilized primarily by hydrophobic interactions, helical wheel diagram generated by DrawCoil 1.0 (https://grigoryanlab.org/drawcoil).
coated chip allowing immobilization of GST-Khc,\(^{848–941}\) \(aTm1^{252–334}\) was added stepwise (fivefold increasing concentration at each step), and the resulting sensogram could be fitted to a standard 1:1 kinetic model with a dissociation constant \(K_d\) of 141 ± 6 nM (Fig. 5B).

The \(aTm1^{252–334}–\text{Khc}^{855–942}\) complex crystallized in a P2\(_1\) space group. A data set was collected to a resolution of 2.3 Å and a model obtained by ab initio molecular replacement with Arcimboldo [Rodríguez et al. 2009]. The structure was refined to a final \(R_{work}/R_{free}\) of 0.24/0.28, incorporating \(aTm1\) (residues 259–324), KhcI (residues 855–923), and KhcII (residues 855–916) in 1:2 \([aTm1:Khc]\) stoichiometry (Fig. 5C). The trimeric structure comprises two parallel, in-register Khc chains and a single, antiparallel Tm1 chain, with the Khc C-terminal residues in close proximity to the \(aTm1\) N-terminal region. This arrangement suggests that the coiled coil of \(aTm1\) opens to accommodate the coiled-coil stalk of Khc. A possible explanation for the mechanism of \(aTm1\)–Khc complex formation would be a lower dimerization propensity of the triple coiled coil. To estimate the \(K_d\) of the \(aTm1\) homodimer, we performed an isotothermal titration calorimetry (ITC) experiment whereby we fitted the heat changes upon dilution of \(aTm1^{252–334}\) [Supplemental Fig. S4A], and obtained a \(K_d\) of 10 µM. Thus, \(aTm1\) has a higher affinity to Khc \([K_d\ in the nanomolar range]\) (Fig. 5B) than to another \(aTm1\), which explains why \(aTm1\) forms a complex with the Khc homodimer.

Superimposition of the \(aTm1\) homodimer on the Khc–\(aTm1\) complex structure revealed no major changes of the \(aTm1\) helix except for a kink caused by the looping out of T277 [Supplemental Fig. S4B]. As crystals of Khc truncations were only obtained in the presence of \(aTm1\), we could not evaluate the effect of \(aTm1\) binding on Khc conformation.

The triple coiled coil is held together by van der Waals forces between mostly hydrophobic residues at “a” and “d” positions of each helix (Fig. 5D). Additionally, the surface of the complex is characterized by a positively charged patch, not present in the \(aTm1^{262–363}\) homo- dimer, formed by the polar side chains of K884, K887, and R886 of Khc [Fig. 5C, right panels], important for RNA binding (see below).

**Mutational analysis of \(aTm1\)–Khc interaction contacts**

To verify the interaction surface of \(aTm1\) and Khc, we generated single point mutants that would be expected to disrupt complex formation. We first mutated two amino acid residues in the unique region of the \(aTm1\) coiled coil, L271 at the hydrophobic core of the complex, and Q278, whose polar side chain is positioned near the hydrophobic core due to a kink in \(aTm1\) [Supplemental Figs. S4B, S5A]. Mutating \(aTm1\) L271 to the smaller nonpolar residue alanine or the bulkier, charged arginine only mildly affected the interaction with Khc, whereas the Q278A mutation had no effect [Supplemental Fig. S5B, left and middle panels]. This might be due to the extensive hydrophobic core of the \(aTm1\)–Khc complex. To achieve disruption of the interaction, we designed two triple mutants—

\(aTm1^{L267A/L271A/L279A}\) [\(aTm1^{T1}\)] at the unique N-terminal extension of \(aTm1\), and \(aTm1^{L296A/L300A/L307A}\) [\(aTm1^{T2}\)] in the middle of the \(aTm1\) coiled coil—and a combination of the two [\(aTm1^{Hexa}\)] (Fig. 6A; Supplemental Fig. S5C). Both the \(aTm1^{T1}\) and \(aTm1^{Hexa}\) mutations fully abolished interaction with Khc, as determined by in vitro binding assays [Supplemental Fig. S5D]. However, although we changed the target residues to alanines in order to preserve the helical propensity of \(aTm1\), circular dichroism (CD) spectroscopy showed that the mutations affected substantially the structure of the \(aTm1\) homodimers [Supplemental Fig. S5E].

As the triple mutant T2 showed residual binding to Khc, we focused on T1 and designed two double mutants, \(aTm1^{L267A/L271A\_L279A}\) [\(aTm1^{D1}\)] and \(aTm1^{L271A/L279A\_L279A}\) [\(aTm1^{D2}\)] (Fig. 6A). The mutations introduced in \(aTm1^{D2}\) substantially reduced the solubility of \(aTm1^{252–334}\) since most of the recombinantly expressed protein precipitated after centrifugation of the E.coli lysate (Fig. 6B). This might be due to the fact that L279 lies at the junction of the two \(aTm1\) chains in close proximity to L314 and A321 of the second chain and is therefore essential for the stability of the \(aTm1\) coiled coil. This again demonstrates how the fact that \(aTm1\) uses the same surface to form a homodimer and to interact with Khc renders it difficult to generate an \(aTm1\) mutant that has no effect on the structure of \(aTm1\) yet disrupts its interaction with Khc. Thus, even the two mutations in \(aTm1^{D1}\) appear to destabilize the coiled coil to some extent, as a SEC-MALS [size exclusion chromatography multilight scattering] measurement indicates that \(aTm1^{D1}\) behaves as a mixture of dimers and monomers [Supplemental Fig. S5F]. Nonetheless, the \(aTm1^{D1}\) mutations did not affect solubility of the protein and had only a moderate effect on its secondary structure as assessed by CD spectroscopy [Fig. 6B,C]. More importantly, \(aTm1^{D1}\) interaction with Khc was notably reduced in our GST pull-down assays and as evaluated by SPR [Fig. 6D; Supplemental Fig. S5G].

To assess \(aTm1^{D1}\) function in vivo, we generated a GFP-tagged \(aTm1^{D1}\) transgene and tested its ability to rescue oskar mRNA localization in \(tm1^{osf}\) oocytes. smFISH revealed that the bulk of oskar mRNA was unlocalized, with only trace amounts detected at the posterior pole [Fig. 6E, Supplemental Fig. S5H], further confirming the importance of the \(aTm1\)–Khc interaction for oskar mRNA transport.

**\(aTm1\) stabilizes the interaction of Khc with RNA**

Previous findings suggest a direct interaction of \(aTm1\) with the oskar 3′ UTR, supporting the notion that \(aTm1\) might function as a Khc adaptor for RNA [Gaspár et al. 2017a]. We therefore tested whether \(aTm1\) interacts with poly [U] RNA in vitro. Considering the rescue of the \(Tm1^{5\_atg3}\) loss-of-function mutant by \(aTm1^{1–334}\) (Fig. 3), we focused our analysis on this portion of the protein. Since recombinantly expressed \(aTm1^{1–334}\) was unstable and gave rise to two proteolytic products [Supplemental Fig. S6A], we generated further truncations of \(aTm1\) to...
Figure 6. Mutational analysis of the aTm1–Khc interaction. (A) Designing double point mutations at the hydrophobic core of aTm1–Khc coiled coil. Ribbon views of aTm1–Khc complex (top panels) or aTm1 homodimer (bottom panels) in two different orientations. Three residues—I267, L271, and L279—at the hydrophobic core of the trimeric complex are highlighted. In aTm1D1, residues I267 and L271 are mutated to alanine. In aTm1D2, residues L271 and L279 are mutated to alanine. (B) aTm1D2 has significantly reduced solubility. Total, supernatant, and pellet fractions of E. coli lysate with recombinantly expressed His-SUMO tagged aTm1252–334 wild-type and mutant variants were analyzed on SDS-PAGE and stained with Coomassie. (C) aTm1D1 has the characteristic coiled-coil fold. (Magenta) CD analysis of aTm1wt, (orange) aTm1L271A, (green) aTm1D1. (D) aTm1D1 mutation causes strong reduction of the interaction with Khc. Pull-down assays of GST-tagged Khc848–942 with different variants of aTm1252–334. (Left panel) Purified aTm1 variants were mixed in excess with Khc848–942 immobilized on GSH beads. (Right panel) After incubation and extensive washing, the eluates were analyzed on SDS-PAGE and stained with Coomassie. (E) aTm1D1 causes oskar mRNA localization defects in vivo. (Left panels) Egg chambers (stage 9) from flies expressing GFP-aTm1-FL and GFP-aTm1D1 in the Tm1eg9/Tm1/eg9 background. (Right panels) oskar mRNA was detected by smFISH. Scale bar, 25 µm. (F) Mean oskar distribution (green) in stage 9 oocytes from the flies in E. n corresponds to the number of analyzed oocytes. (G) Position of oskar mRNA center of mass relative to the geometric center of the oocyte, represented in box plot with minimum and maximum whiskers. GFP-aTm1FL wt panels and quantification in E–G are the same as in Figure 3.
probe its interaction with RNA. Previously we showed by NMR, which allows detection of stable as well as transient interactions, that Tm11–246, which encompasses most of the low complexity N-terminal region of aTm1, interacts directly with U25 RNA [Vaishali et al. 2021]. Moreover, electrophoretic mobility shift assays (EMSA) showed that the C-terminal region of Tm11–334, Tm1252–334, binds oligo-U25 only weakly, with a \( K_d \) >10 \( \mu \)M, as compared with Tm154–335, which demonstrated significantly higher affinity for RNA [Fig. 7A]. These findings suggest that a major RNA binding activity of aTm1 lies within the N-domain (residues 1–246).

As deletion of the N domain of aTm1 had only a mild effect on oskar localization [Fig. 3; Supplemental Fig. S3], we suspected the presence of an additional RNA binding moiety within the Khc–aTm1 complex. Indeed, as mentioned above, the structure of the aTm1–Khc complex displays a joint positively charged surface [Fig. 5C]. We therefore performed EMSAs with Khs855–941 alone or in complex with aTm1. Khc on its own bound RNA with high affinity (high nanomolar range) and, at higher concentrations, appeared to form oligomers with RNA that failed to enter the gel [Fig. 7B]. Upon addition of Tm1252–334 or Tm154–335 to Khc in a 1:2 ratio, the affinity increased and the protein–RNA complexes could be resolved [Fig. 7B]. The affinity increase for Tm154–335, as expected, has a stronger effect, as the N domain contributes to RNA binding. As an independent method to assess the interaction of aTm1–Khc with RNA, we performed filter binding assays, which overall confirmed the EMSA results showing that the interaction of Khc with RNA in solution is enhanced almost to the same extent by Tm1252–334 and Tm154–334 [Fig. 7C]. Together, these data show that aTm1 prevents formation of Khc–RNA aggregates and that the aTm1 and Khc synergize in RNA binding.

To ascertain that the interaction of Khc with RNA is not due to stickiness of the Khc855–941 truncation, we also performed EMSAs with the full-length Khc, alone or in the presence of Tm1252–334 or Tm154–335. This confirmed that Khc-FL binds to RNA and that its affinity for RNA increases when aTm1 is present [Supplemental Fig. S6B].

The Khc855–941 fragment used in our RNA binding and crystallization experiments contains both the aTm1 binding site (residues 855–908) and the ATP-independent microtubule binding site [AMB, residues 910–938]. To determine which of the two regions mediates RNA binding, we tested two further truncations from which the AMB was either partially or fully deleted (Khc855–930 and Khc855–909). Both proteins, and especially Khc855–909, showed reduced binding to RNA [Supplemental Fig. S6C]. To test for involvement of the positively charged patch on the surface of aTm1–Khc in RNA binding, we mutated Lys884, Lys887, and Arg888 of Khc to alamines [Khc855–941/A] [Supplemental Fig. S6D]. This reduced the binding of Khc to RNA but had no effect on protein folding as measured by CD spectroscopy or on binding to aTm1 as assessed by a GST pull-down assay [Supplemental Fig. S6E–G]. These experiments show that both the aTm1 binding site and the AMB contribute to RNA binding, most likely by formation of an extended RNA interaction surface. These data are consistent with the finding of Williams et al. [2014] that Khc1–910, which lacks the auxiliary microtubule binding domain, does not support oskar mRNA localization.

Taken together, our findings demonstrate that Khc can bind RNA directly and that aTm1 acts in stabilizing rather than in mediating the Khc–RNA interaction, distinguishing aTm1 from a classical kinesin adaptor.

**Discussion**

In recent decades, mechanisms of mRNA transport and the composition of mRNP transport complexes have been extensively studied, yet only a few adaptor proteins linking RNAs to the transport machineries have been identified. These include She2p/She3p for myosin-based RNA transport in *S. cerevisiae* [Takizawa and Vale 2000], and Egalitarian/BicD proteins for dynenin-based transport in *Drosophila* [Dienstbier and Li 2009; Dienstbier et al. 2009]. The atypical tropomyosin isoform aTm1 was recently proposed to function as an adaptor for Khc-mediated transport of oskar mRNA in *Drosophila* [Veeranankarmegam et al. 2016; Gaspár et al. 2017a]. Classical Tm1 isoforms form parallel coiled coils that polymerize in head-to-tail fashion along actin filaments and are known to regulate different functions of the actin cytoskeleton [Parry and Squire 1973; Gunning et al. 2015]. aTm1, which is present in Drosophilidae, has a shortened coiled-coil region flanked on both sides by unique, intrinsically disordered N-terminal and C-terminal sequences. Crystal structures of the aTm1 coiled coil presented in this study reveal an antiparallel conformation with a coiled-coil region extended by 20 amino acids beyond the conserved region [Fig. 4]. Coiled coils play vital roles in nearly all biological processes and are among the most studied structural motifs [Truebestein and Leonard 2016]. A few point mutations in the hydrophobic core are sufficient to cause a switch in the orientation of the coiled coil or the number of coils involved in the interaction [Macon and Arndt 2004; Malashkevich et al. 2015]. Therefore, it is likely that the unique extension of the aTm1 coiled-coil region causes the antiparallel topology of the two chains. Importantly, two point mutations in this region have a dramatic effect on aTm1’s interaction with Khc, which suggests that the unique sequence contributes to defining the specificity of aTm1 for Khc, both at the secondary and tertiary structure level (Fig. 6). Additionally, the switched orientation of the aTm1 coiled coil prevents interaction of the protein with actin and thus prevent its interference with the function of the classical actin-binding tropomyosin isoforms [Cho et al. 2016].

Although we could not determine a structure of the Khc dimer on its own, we assume that its conformation does not change significantly when bound to aTm1. This is corroborated by rotary shadowing negative stain EM images of Khc, which show that the motor domains are symmetrically attached to a long rod, a conformation that can be achieved only when Khc is a parallel in-register coiled coil [Hirokawa et al. 1989]. The aTm1 homodimer
**Figure 7.** αTm1 as a kinesin modulator. (A) αTm1-N domain binds to RNA. Electrophoretic mobility shift assay (EMSA) of αTm1<sup>252–334</sup> and αTm1<sup>54–335</sup> variants. <sup>32</sup>P-labeled oligo-[U25] was incubated with twofold increasing concentrations [0.15, 0.3,..., 10 μM] of recombinantly purified proteins in 15-μL reactions as indicated. The samples were then loaded on 6% native gel and visualized by autoradiography. Free probe was run in the first lane. (B) Khc binds to RNA alone and better in the presence of αTm1. Electrophoretic mobility shift assay (EMSA) of Khc<sup>855–941</sup> alone and in the presence of αTm1<sup>252–334</sup> (left gel) or of αTm1<sup>54–335</sup> (right gel). For the complex, αTm1 was added to Khc in 1:2 molar ratio prior to performing serial dilutions. (C) Nitrocellulose filter binding assay of αTm1 and Khc variants alone and in complex. <sup>32</sup>P-labeled oligo-[U25] probe was incubated with increasing concentrations of proteins and protein complexes. The mixture was then passed through a nitrocellulose filter, and the radioactive probe left on the filter was measured as a fraction of the total probe that was added to the reaction. (D) Hypothetical model of αTm1 as a modulator of Khc interaction with RNA. αTm1 and Khc each bind RNA weakly on their own. To accommodate the Khc coiled coil, the αTm1 homodimer opens, allowing formation of a tripartite coiled coil composed of one αTm1 and two Khc chains. The second αTm1 might be rearranged and promote formation of higher order structures. Binding of αTm1 to Khc might elicit conformational changes in Khc or, alternatively, stabilize the Khc RNA binding surface and thus allow the Khc-αTm1 complex to interact with higher affinity and/or specificity with RNA. Since the region with which Khc interacts with RNA incorporates both the alternative cargo binding domain and the adjacent auxiliary microtubule binding domain, the binding of αTm1 to Khc might regulate the kinetic activity of Khc.
coiled coil, on the other hand, must open to allow binding to the kinesin homodimer, thus posing the question of what happens to the second αTm1 chain [Fig. 7D]. It might form an individual complex with another Khc homodimer or, alternatively, rearrange such that it remains bound to the portion of the αTm1 coiled coil not in complex with Khc. This arrangement can further provide a platform for the formation of higher-order structures.

The interaction of Tm1 with Khc involves the alternative cargo binding region of Khc, which lies immediately downstream from the Klc binding domain [Fig. 1]. This region was initially identified as cargo binding in the fungus N. crassa and was subsequently shown to be the major binding site for other adaptors and their respective cargos (Diefenbach et al. 2002, 2004; Setou et al. 2002; Kanai et al. 2004; Glater et al. 2006). We have shown that deletion of this region in Drosophila Khc results in oskar mislocalization and female sterility [Fig. 2], a phenotype more severe than that of complete loss of αTm1, in which case the eggs still hatch and develop into adult sterile flies. This suggests that the alternative cargo binding region of Khc might have functions beyond oskar mRNA localization also in Drosophila. For example, it has been shown that the mitochondrial adaptor Milton binds also to the alternative cargo binding domain, suggesting that in Khc, AM55–911 the mitochondrial transport to and within the oocyte might also be affected (Cox and Spradling 2006). Moreover, Milton along with SNAP25, which is an adaptor for vesicular transport, and the ribosome receptor p180 appear to bind Khc also via coiled-coil interactions, suggesting a binding mechanism similar to the αTm1–Khc interaction described here [Diefenbach et al. 2002, 2004; Glater et al. 2006; Randall et al. 2013].

Functionally, coiled coils allow the formation of long rigid structures, especially when they involve more than two chains, and they may play the role of “molecular rulers,” spacing the different functional regions of a protein (Truebestein and Leonard 2016). Thus, with the Klc binding site and the alternative cargo binding region adjacent to one another and potentially prone to steric hindrance, the coiled-coil structure might provide space for the simultaneous binding of cargos to these two regions. It will be interesting in the future to determine whether different cargos can indeed be cotransported by a single Khc molecule.

Retrograde transport by different kinesins along microtubules comprises a major fraction of the intracellular transport of mRNP granules. Surprisingly however, there are to date scarce mechanistic details of how the cargo might be recognized and how it can be attached to Khc. Only recently, the transport of β-actin and β2B-tubulin mRNAs was reconstituted in vitro, whereby APC [adenomatous polyposis coli] links the mRNAs to kinesin-2 through the cargo adaptor KAP3 (Baumann et al. 2020). Another study suggests that the transport of some axonal mRNPs depends on KLC1, which also implies a more classical adaptor-based transport mechanism (Fukuda et al. 2021). Here, we have shown that the N domain [residues 1–246] of αTm1 binds RNA, whereas the region immediately adjacent to it [residues 247–334] interacts with the alternative cargo binding region of Khc [Figs. 1, 7]. Therefore, it is appealing to imagine αTm1 as an adaptor for Khc as well. However, deletion of the N domain in vivo resulted in only minor oskar mRNA mislocalization, and sole expression of the Khc binding domain almost restored fully the accumulation of oskar at the posterior pole of the oocyte [Fig. 3]. As demonstrated, Khc binds to RNA also on its own, and its RNA affinity increases in the presence of αTm1 [Fig. 7]. Moreover, the Khc-RNA interaction appears to be mediated by both the αTm1 binding surface and the adjacent ATP-independent microtubule binding region [Supplemental Fig. S6]. This explains the strong effect of deletion of the ATP-independent microtubule binding region on oskar mRNA localization (Williams et al. 2014). Therefore, αTm1 appears to act as a stabilizer of the Khc interaction with RNA rather than as a classical adaptor [Fig. 7].

It is possible that αTm1, upon binding, induces a conformation change of Khc, leading to increased RNA binding. This is supported by the structure reported here, as part of the AMB site of Khc appears to acquire a helical conformation [residues 909–920], although predicted to be disordered. Despite the lack of an isolated Khc structure for comparison, it can be speculated that the binding of αTm1 stabilizes the helical conformation of the alternative cargo binding domain that extends into the AMB site and thus enables a higher-affinity interaction of Khc with RNA. Interestingly, downstream from AMB [residues 938–950] (Williams et al. 2014) is the Khc IAK autoinhibitory motif, which interacts with the motor domain of Khc and keeps the protein inactive in the absence of cargo (Kaan et al. 2011). Because of the antiparallel topology of the αTm1 chain within the αTm1–Khc complex, the N domain of αTm1 is in proximity to the IAK motif of Khc. Thus, besides enabling the binding of cargo to Khc, αTm1 might also play a role in release of the autoinhibited conformation of Khc. This might explain the observation that, upon deletion of the N domain of αTm1, most but not all oskar localizes to the oocyte posterior pole [Fig. 3, Supplemental Fig. S3].

Our high-resolution structure of the atypical Tm1 [αTm1] suggests that the unique antiparallel coiled-coil conformation of its dimers might underlie the specific functions of this isoform. Together with our high-resolution structure of the complex of αTm1 with Khc, our findings show that αTm1 stabilizes the interaction of Khc with RNA. Whether αTm1 binding increases Khc’s specificity for oskar mRNPs alone or through additional protein-protein or protein-RNA interactions in vivo remains to be investigated. Future identification of the αTm1 interactome and of the oskar mRNA sequence or structure to which the αTm1–Khc complex binds will be essential to elucidate the full mechanism whereby this RNA transport complex recognizes its different cargos.

Materials and methods

Plasmid construction

Cloning techniques including digestion, ligation, and DNA electrophoresis were performed essentially as described in Sambrook
et al. [1989] unless indicated otherwise. Mutagenesis was performed according to Liu and Naismith [2008]. All GST-tagged Khc constructs [Khc1-264, KhcA265-975, KhcA1-345, KhcA345-848, KhcA284-941, KhcA1-475, KhcA475-941, KhcA549-941, KhcA523-941, and KhcA86-941] were cloned between BamHI and EcoRI sites of the pHIS6p-1 vector [Amersham]. All His$_6$-SUMO tagged Tm1 (Tm1-FL, Tm1A1-355, Tm1A247-378, Tm1A1-246, Tm1A247-334, and Tm1A335-441) and Khc (KhcA585-941, KhcA685-909, and KhcA685-926) truncations were cloned between BamHI and SalI sites of the pETM11-His$_6$-SUMO plasmid [derived from pBR322, H. Besir, Protein Expression and Purification Core Facility, EMBL Heidelberg]. The simple modular architecture research tool [SMART] was used for designing the truncation boundaries (Letunic and Bork 2018).

For yeast two-hybrid assays, Khc-FL, KhcA265-975, and KhcA685-975 were cloned between NdeI and BamHI sites of the pG4BDN22 vector, whereas Tm1-FL, Tm1A1-247, and Tm1A247-441 were cloned between NdeI and BamHI sites of the pG4ADHAN111 vector (Thoms et al. 2015).

The Tm1 integrase constructs were based on pUASP2-emGFP-Tm1RI [Gáspár et al. 2017a]. Essentially, the αTm1 open reading frame [ORF] and 3′ UTR were cloned between BamHI and BglII sites of the pUAS vector [Rurth 1998]. emGFP was inserted downstream from the pUAS promoter in-frame with the Tm1 coding sequence. To generate pUASP-attB-ΔK10-Tm1 constructs, the region of pUASP-emGFP-Tm1 containing the pUAS promoter, emGFP, Tm1-FL, or truncations’ ORFs, and Tm1 3′ UTR region were PCR-amplified and inserted between NotI and XbaI sites of pUAS-attB-ΔK10 plasmid using InFusion cloning according to the manufacturer’s instructions [Takara].

To generate the pHkp-attB-ΔK10-Khc-FL construct, we amplified the 2R-16266960-16273471 region from fly genomic DNA and inserted it between EcoI and BamHI sites of pUASP-attB-ΔK10 vector, removing the pUAS promoter. We then performed site-directed mutagenesis using the following oligos: 5′-CGCTTGTTGTTGATATTGCTGAGATGTC-3′ (fwd) and 5′-TTATACGAGGTAGCTGAGCTGTTGGCAAGAGCA-3′ (rev) to change the region targeted by Khc-RNAi [Bloomington Stock 35409]. The mKate2 coding sequence was added in-frame at the C terminus of the Khc ORF using the ligation-free cloning approach. To generate the pUASP-attB-ΔK10-KhcA885-911 truncation, we performed inverse PCR with oligos starting at the boundaries of the truncation, followed by ligation of the product and transformation in E. coli.

Khc-FL was cloned with a His$_6$-SUMO-SNAP-tag between BamHI and HindIII sites of the pFastBacDual vector. All plasmids used in this study are summarized in Supplemental Table S2.

**Protein expression and purification**

GST- and His$_6$-SUMO-tagged proteins were expressed in BL21-CodonPlus(DE3)-RIL cells [Stratagene] by isopropyl β-D-1-thiogalactopyranoside (IPTG) induction for 16 h at 18°C. Cells were grown in Luria-Bertani (LB) medium (0.5% [w/v] yeast extract [MP], 1% [w/v] tryptone [MP], 0.5% [w/v] NaCl at pH 7.2) supplemented with antibiotics [100 μg/mL ampicillin or 10 μg/mL kanamycin and 34 μg/mL chloramphenicol]. After harvesting, the pellets were lysed by a microfluidizer processor [Microfluidics] in 500 mM NaCl, 20 mM Tris-HCl [pH 7.5], 5% glycerol, 0.01% NP-40, and 40 mM imidazole [only in the case of His$_6$-tagged proteins] buffer supplemented with protease inhibitor cocktail [Roche] and 5 mM β-mercaptoethanol. The lysates were subsequently cleared by centrifugation at 18,000 rpm for 20 min.

For GST-tagged proteins, the lysates were incubated with glutathione sepharose 4B [GE Healthcare] for 1 h at 4°C. After washing, the beads were directly used for binding assays (see below).

His$_6$-SUMO tagged proteins were affinity-purified by a His TRAP Ni column [GE Healthcare] and eluted over an imidazole gradient (40–600 mM). After cleavage of the tag by Senp2 protease, the proteins were passed one more time over a His TRAP Ni column to remove the tag and protease. Finally, the eluates were further separated on a Superdex 200 16/600 column in a 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.5 mM TCEP buffer for crystallization and binding assays and 20 mM HEPES-Na [pH 7.5], 150 mM NaCl, and 0.5 mM TCEP for surface plasmon resonance (SPR) and CD spectroscopy.

His$_{6}$-SUMO-SNAP-tagged Khc-FL was expressed in 1 L of SF-21 insect cells at 1 × 10^{6} cells/mL shaking culture infected with 10 mL of P1 virus stock. After expression for 3 d at 27.5°C, cells were harvested by centrifugation at 1000g for 20 min at 4°C. The pellet was lysed in a dounce tissue grinder in lysis buffer (20 mM Tris/HCl at pH 7.5, 500 mM NaCl, 1 mM MgCl$_2$, 0.1 mM ATP, 2 mM DTT, 5% glycerol, 0.2% Tween-20). The lysate was cleared by centrifugation and the soluble protein fraction was affinity-purified on a HisTrap Excel column [GE Healthcare]. After elution in a 0–300 mM imidazole gradient, the His$_6$-SUMO-SNAP-fusion tag was cleaved by 3C protease digest upon dialysis in 25 mM HEPES/KOH [pH 7.3], 150 mM KCl, 1 mM MgCl$_2$, 0.1 mM ATP, and 2 mM DTT for 16 h before further purification by anion exchange chromatography on a HiTrap Q HP column [GE Healthcare], followed by size exclusion chromatography [SEC] on a Superdex 200 10/300 Increase column [GE Healthcare].

For expression of seleno-methionine (SeMet)-labeled Tm1[334–352], we used the B834(DE3) methionine auxotrophic E. coli strain. After transformation, the cells were grown in minimal M9 medium containing 50 μg/mL methionine overnight at 37°C. The bacteria were then scaled up to the volume of the expression culture and grown to OD$_{600}$ = 1 in minimal medium supplemented with methionine. The cells were then harvested, resuspended in minimal medium without methionine, and starved for 4–8 h before the addition of 50 μg/mL SeMet to the culture. After 30 min, protein expression was induced with 200 mM IPTG for 16 h at 18°C [for a detailed protocol see https://www.embl.de/pepcore/pepcore_services/protein_expression/ecoli/

**Yeast two-hybrid assay**

The yeast two-hybrid analysis was performed according to James et al. [1996]. The yeast two-hybrid strain PJ69-4a was transformed with the indicated αTm1 or Khc fragments cloned in pAS or pG4ADHAN vectors [Clontech Laboratories, Inc.], and plated on SDC–TRP–LEU or SDC–TRP–LEU–His plates. A positive interaction was monitored by growth on SDC–TRP–LEU–His plates. pTD1-1 [Clontech Laboratories, Inc.], which contains pGAL4-AD SV40 large T-antigen, and pVA3-1 [Clontech Laboratories, Inc.], which contains pGAL4-BD-tagged murine p53, were used as positive controls.

**GST pull-down assays**

Proteins for pull-down assays were purified in 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5% glycerol, and 0.01% NP-40 binding buffer. Baits were purified on glutathione sepharose beads [see above]. Lysates from E. coli expressing the protein of interest, E. coli lysates mixed with prepurified bait proteins or pure proteins were added with bait protein five times in excess of the prey protein, immobilized on GSH beads. After incubation on a turning wheel at 4°C and extensive washing, the proteins were eluted in sample buffer [200 mM Tris-HCl at pH 6.8, 8% [w/v] SDS, 40% glycerol, 0.4% bromophenol blue, 100 mM DTT] for
30 sec at 90°C. The eluates were analyzed by SDS-PAGE and stained with Coomassie.

**Crystallization, data collection, and structure determination**

Tm1<sup>262–363</sup> was concentrated to 36 mg/mL and mixed in 1:1 ratio (100 nL:100 nL) with 0.1 M Tris-HCl (pH 8) and 40% MPD reservoir solution at 20°C. Rod-shaped crystals were obtained by sitting drop vapor diffusion method between day 21 and 35. Crystals were harvested in mother liquor without additional cryoprotectant. Tm1<sup>270–334</sup> produced needle-like crystals 1 d after setting the hanging drops at 20 mg/mL in 1:1 ratio with 0.1 M NH₄Ac (pH 4.6) and 15% [w/v] PEG 4000 reservoir solution. The crystals were harvested in mother liquor supplemented with 25% glycerol.

Tm1<sup>252–334</sup>/Khc<sup>855–942</sup> complex crystals were obtained after mixing the SeMet-labeled Tm1<sup>252–334</sup> and unlabeled Khc<sup>855–942</sup> proteins in 1:1 ratio followed by further purification by size exclusion chromatography. The peak fractions corresponding to the complex were concentrated to 4.5 mg/mL and mixed in 1:1 ratio (100 nL:100 nL) with 0.2 M NaAc, 20% PEG 3350. Thin plate-shaped crystals were obtained after 1 d, and crystals were harvested in mother liquor supplemented with 25% glycerol.

Diffraction data sets were recorded at ID-29 beamline at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Data were processed with XDS [Kabsch 2010], and the structures were solved by ab initio molecular replacement with AmoRe [Bibby et al. 2012] or Archimboldo [Rodriguez et al. 2009]. Model building and several cycles of refinement were performed with Coot [Emsley et al. 2010] and with the Phenix suite [Liebschner et al. 2019], respectively.

The crystal structures are deposited at the Protein Data Bank under the following accession codes: Tm1<sup>262–363</sup> [7BG], Tm1<sup>270–334</sup> [7BN], and Tm1<sup>252–334</sup>/Khc<sup>855–942</sup> complex [7BJ].

**Circular dichroism spectroscopy**

The proteins were purified in 20 mM HEPES/NaOH (pH 7.5), 150 mM NaCl, and 0.5 mM TCEP for circular dichroism (CD) spectroscopy. Prior to measurement, the salt was diluted to 50 mM NaCl. The measurements were performed at a 20 µL concentration in a 0.2-mm cuvette at 20°C using a Jasco J-815 CD spectrophotometer. The monitored wavelength range was from 240 to 190 nm in 0.1-nm steps with an average of five points per wavelength. The proteins were purified in 20 mM HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM TCEP. The Tm1 WT protein in the syringe, at a concentration of 1 mM, was injected into the cell containing the buffer. Titrations were performed at 20°C or 25°C and consisted of 13 or 25 injections. The data were fitted using the dissociation fitting model of the MicroCal PEAQ-ITC analysis software (Malvern).

**Fly stocks and husbandry**

Tm1 and Khc transgenic flies were generated by site-specific integration of the respective pUA53-attB-AK10 or pKhc-attB-AK10 constructs (see above) with Fs21 integrase in VK18 (vas-phiZH2A, PBac[+]/-[attB-A9]/VK00018, Bellen laboratory) fly line. emGFP-Tm1–FL, truncations, and point mutations were driven by one copy of oskGal4 [Tetley et al. 2012], FBP0083699 in the Tm1<sup>93</sup>/Tm1<sup>99</sup> background [Falou049223] [Erdélyi et al. 1995]. Khc–FL-mKate2 and Khc<sup>855–911</sup>–mKate2 were constitutively expressed by the Khc endogenous promoter in the UASp-Khc RNAi Trip Line GL00330 [Staller et al. 2015] background where the expression of Khc-RNAi was driven by oskGal4.

All fly stocks were grown at 21°C–25°C in vials on standard cornmeal agar. Prior to dissection, freshly hatched flies were fed with dried yeast for 1–2 d.

**Single-molecule fluorescent in situ hybridization (smFISH)**

Forty-two probes against the oskar mRNA coding region and 3′ UTR were labeled with Atto633 according to Gáspár et al. (2017b). smFISH was performed essentially as in Gáspár et al. (2017a). In short, two to three pairs of Drosophila ovaries were fixed with 2% [v/v] PFA and 0.05% [v/v] Triton X-100 in PBS (pH 7.4) for 20 min on an orbital shaker, followed by two washes with PBT [PBS + 0.1% [v/v] Triton X-100 at pH 7.4] for 10 min each. Ovaries were then prehybridized in 100 µL of hybridization buffer [500 mM NaCl, 30 mM sodium citrate at pH 7.0, 15% [v/v] ethanolamine carbonate, 1 mM EDTA, 50 µg/mL heparin, 100 µg/mL salmon sperm DNA, 1% [v/v] Triton X-100] for 30 min at 42°C, to which 100 µL of probe mixture (25 nM per individual oligonucleotide in hybridization buffer) was added for an additional 2–3 h at 42°C. After hybridization, the following washes were carried out to remove excess probes: hybridization buffer, hybridization buffer to PBT in a 1:1 mixture, PBT [10 min at 42°C each], and PBT at room temperature. The samples were then mounted in 80% [v/v] 2,2-thiodiethanol (TDE) in PBS and viewed using a Leica TCS SP8 confocal microscope with a 63× 1.4 NA objective. To detect GFP-tagged Tm1 variants, the native fluorescence of GFP was visualized. Because the mKate2 tag was not visible after smFISH, we imaged the ovaries directly after fixation in 2% PFA/PBT for 20 min. The images were analyzed in ImageJ/Fiji. Analysis of the oskar mRNA center of mass distribution was performed according to Gáspár et al. (2014) and plotted with GraphPad Prism 5.

**Immunological techniques**

For Western blots, dissected ovaries were lysed in 2× SDS sample buffer [100 mM Tris-HCl at pH 6.8, 4% [w/v] SDS, 20% glycerol,
0.2% bromophenol blue, 50 mM DTT) by manual grinding and heated for 10 min at 85°C. After separation by SDS-PAGE, the lysates were blotted on Immobilon-P PVDF membrane (Millipore) by wet blot transfer for 2 h at 4°C and 80 V. After blocking in 5% milk/PBS-Tween, incubation with primary antibody for 16 h and secondary horse radish peroxidase (HPR)-conjugated antibody for 1 h, the blot was developed with Immobilon Western chemiluminescent HPR substrate (Millipore).

The anti-Khc antibody was raised against the Khc345–599 truncation in rabbits.

**Electrophoretic mobility shift assays (EMSAs)**

The U25 RNA synthetic probe (IDT) was 5′ end-labeled with ATP, [γ-32P] (Hartmann Analytic) using T4 polynucleotide kinase (Thermo Fisher) and subsequently purified using Illustra microspin G25 columns (GE Healthcare). RecombinantTrim10 and Khc were mixed with 2.5 nM probe in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% glycerol, and 0.5 mM TCEP binding buffer in 15-µL reactions and incubated for 45 min on ice. The samples were subsequently separated on 5% native 0.5× TBE polyacrylamide gel for 1 h at 100 V. The gel was then dried and exposed overnight to a storage phosphor screen (GE), which was visualized with a Typhoon Trio Imager (GE Healthcare).

**Filter binding assays**

For the filter binding assays, 2 CPS/µL (counts per second) radiolabeled probe was mixed with protein or protein mixture in a 100-µL reaction volume. After incubation for 45 min on ice, the mixture was filtered through Whatman 0.45-µm pore size nitrocellulose filters. Only protein-RNA complexes were retained on the filters and detected by scintillation counting. Data fits were performed with GraphPad Prism 5 using a single site saturation binding model.

**Competing interest statement**

The authors declare no competing interests.

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**Author contributions**

L.D.-P., J.H., and A.E. conceived the study and wrote the manuscript. P.K.A.J. helped with data collection and solved the crystal structures. V. and A.C. helped with cloning, protein purifications, and fly husbandry. K.L. performed the ITC and SEC-MALS experiments. P.S. carried out the biacore experiments. K.P. measured the CD spectra. S.H. provided the full-length Khc. L.D.-P. carried out the rest of the experiments and data analysis. C.L. provided laboratory space and resources. All authors commented on the manuscript.

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