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Report

Guidance of Bidirectional Motor Complexes by mRNA Cargoes through Control of Dynein Number and Activity

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Summary

During asymmetric cytoplasmic mRNA transport, *cis*-acting localization signals are widely assumed to tether a specific subset of transcripts to motor complexes that have intrinsic directionality. Here we provide evidence that mRNA transcripts control their sorting by regulating the relative activities of opposing motors on microtubules. We show in *Drosophila* embryos that all mRNAs undergo bidirectional transport on microtubules and that *cis*-acting elements produce a range of polarized transcript distributions by regulating the frequency, velocity, and duration of minus-end-directed runs. Increased minus-end motility is dependent on the dosage of RNA elements and the proteins Egalitarian (Egl) and Bicaudal-D (BicD). We show that these proteins, together with the dynein motor, are recruited differentially to different RNA signals. Cytoplasmic transfer experiments reveal that, once assembled, cargo/motor complexes are insensitive to reduced cytoplasmic levels of transport proteins. Thus, the concentration of these proteins is only critical at the onset of transport. This work suggests that the architecture of RNA elements, through Egl and BicD, regulates directional transport by controlling the relative numbers of opposite polarity motors assembled. Our data raise the possibility that recruitment of different numbers of motors and regulatory proteins is a general strategy by which microtubule-based cargoes control their sorting.

Results and Discussion

Localization Signals Modulate the Directionality of Bidirectional Motor Complexes

mRNA localization signals have recently been found to modulate the kinetics of microtubule-based transcript movements [1, 2]. In mammalian cells, uniformly distributed mRNAs undergo short, unidirectional transport events that are augmented in duration and frequency by the RNA signal from the localizing *β-actin* transcript [1]. In *Drosophila* syncytial blastoderm embryos, individual nucleotide changes in the *hairy* (*h*) transcript slow the rate of delivery of injected fluorescently labeled mRNAs to the apical cytoplasm [2]. However, it is unclear whether localization elements simply limit dissociation of mRNAs from their RNA binding protein(s), and hence the microtubule, or actively regulate motor movement.

To investigate whether mRNA cargoes regulate the movement of motors on microtubules, we have used improved microscopy and automatic tracking software to analyze the detailed movements of mRNAs in the *Drosophila* blastoderm. Here, the microtubules have a stereotypical arrangement, with minus ends nucleated apically and plus ends extending basally (Figure S1A in the Supplemental Data available with this article online).

We observe that particles of injected, fluorescently labeled *h* mRNA are transported bidirectionally, undergoing relatively long runs in the minus-end direction interspersed with shorter reversals (plus-end runs), as well as periods of little or no persistent movement (pauses) (Movie S1; Figure 1A). The net rate of apical transport of the *h* mRNA particles is ~150 nm/s (Figure 2A), with active transport in both directions reaching velocities of up to ~1–1.5 μm/s (data not shown).

The characteristics of mRNA motion are reminiscent of those of other bidirectional cargoes [3, 4]. Firstly, the distances of runs in both directions approximate a decaying exponential distribution (Figure 1B), as if each opposing motor activity ceases productive cargo transport due to a constant-probability event. Secondly, particles frequently undergo rapid switching between minus- and plus-end motility. This suggests that the mRNA binds opposite polarity motors at all times, with the net distribution determined by differences in their relative activities.

It is not clear how control of net transport of other bidirectional cargoes is achieved, although opposing motor activities appear to be mutually dependent [3, 4]. Indeed, inhibition of the minus-end-directed motor dynein, which is required for apical mRNA localization [5], by preinjection of anti-dynein intermediate chain (Dic) antibodies or the dynein inhibitor vanadate, inhibits motility of *h* mRNA in both directions (Movie S2; Figures 2B and 2C; Table S1). The identity of the motor engaged during plus-end movement is not known, and it could even be dynein, as recent work suggests that the motor with its accessory complex dynactin can undergo bidirectional motion on a single microtubule in vitro [6].

To address the mechanistic role of apical localization signals, we contrasted the behavior of *h* transcripts to those of *Krüppel* (*Kr*), which are distributed evenly in the cytoplasm (Figure 1C), or several heterologous mRNAs such as those derived from transcription of a plasmid backbone. Neither the *Kr* nor heterologous mRNA populations become enriched apically following injection, but each of these mRNAs rapidly assembles into particles with a spectrum of sizes similar to those of *h* (Movie S3). Surprisingly, the nonlocalizing

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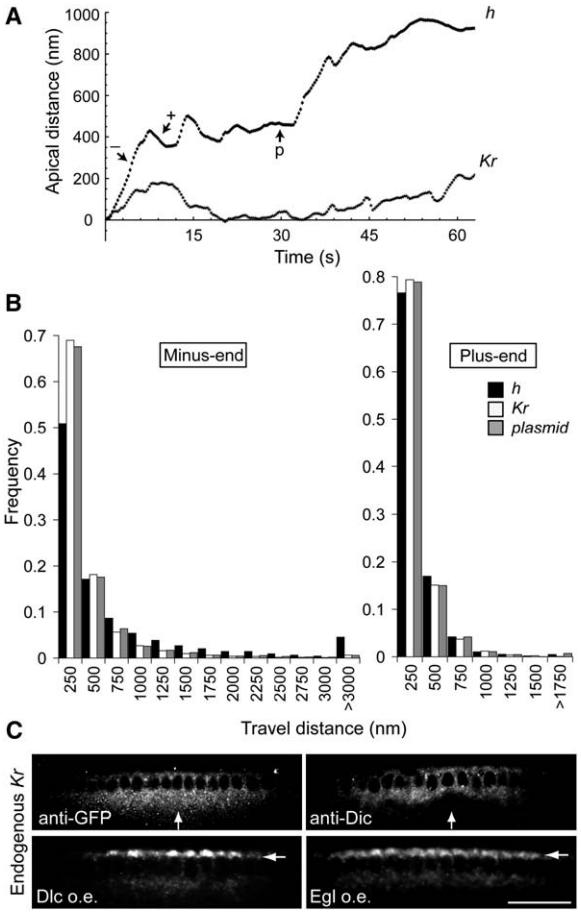

Figure 1. Localizing and Nonlocalizing mRNAs Undergo Bidirectional Transport

(A) Example tracks of localizing (*h*) and nonlocalizing (*Kr*) mRNA particles. p, pause; −, minus-end run; +, plus-end run.

(B) Distribution of travel distances of minus-end- and plus-end-directed runs of localizing and nonlocalizing mRNAs (bins of 250 nm). *h* RNA exhibits more relatively long runs, although plus-end run lengths are not affected by transcript identity.

(C) Inhibition of dynein activity by injection of anti-Dic, but not control anti-GFP antibodies, leads to retention of endogenous nonlocalizing *Kr* transcripts near their site of synthesis in the nuclei. Arrow indicates approximate injection site. Bottom panels, enrichment of *Kr* in the apical cytoplasm (arrow) upon overexpression (o.e.) of Dynein light chain (Dlc) or Egl (weak apical *Kr* localization was found in 40/48 and 27/29 of Dlc and Egl overexpression embryos, respectively, compared to 8/63 wild-type embryos). Scale bar, 50 μm.

transcripts undergo short bidirectional movements that initiate with similar kinetics to localizing mRNAs (Figure 1A; Movie S3).

The movements of the nonlocalizing transcript populations are indistinguishable from one another and are clearly motor driven; like those of *h*, persistent movements in both directions frequently reach velocities of ~1–1.5 μm/s (data not shown) and are sensitive to anti-Dic injection (Figure 2C), as well as to hypomorphic mutations in the gene encoding dynein heavy chain (*dhc64C*; Table S1). Consistent with a physiological function of bidirectional transport in achieving uniform spreading of mRNAs, endogenous *Kr* transcripts are retained in the perinuclear region upon injection of anti-Dic antibodies (Figure 1C). Transport of uniform mRNAs presumably facilitates encounters with other posttranscriptional machinery [1].

The ability of localizing and nonlocalizing mRNAs to undergo bidirectional transport led us to examine which aspects of motion are significant for determining their different net distributions. Compared to nonlocalizing transcripts, apically localizing mRNAs spend more time undergoing minus-end transport and less time moving in a plus-end direction or pausing (Table S1). There is a ~2.5-fold increase in the mean distance of minus-end runs of *h* compared to nonlocalizing mRNAs (Figure 2B). This does not reflect increased dissociation of nonlocalizing mRNAs from recognition factors; for all RNAs tested, the distribution of plus-end run lengths is indistinguishable (Figure 1B; data not shown). Furthermore, minus-end runs are often immediately followed by reversals (Figure 2D), which implies that mRNAs remain associated with a microtubule.

Localizing mRNAs also have a subtle, but significant, increase in mean velocity of minus-end transport (10%–15%) compared to nonlocalizing RNAs, whereas plus-end velocity is not significantly different for all mRNAs tested (Table S1). Localizing mRNAs are also ~1.4 times more likely than nonlocalizing mRNAs to undergo a minus-end run instead of a plus-end run following a pause (Figure 2D). In contrast, the likelihood of a minus-end run or pause following a plus-end run is similar for localizing and nonlocalizing mRNAs (Figure 2D).

Overall, these data indicate that the *h* RNA localization signal is not obligatory for linking mRNAs to molecular motors. Instead, it gives rise to net apical localization by increasing the probability of initiation and maintenance of rapid minus-end-directed excursions of a bidirectional motor complex.

To address whether localization signals are binary switches, we tested the effects of altering the sequence of the *h* element upon mRNA movement. The weak localizing *h* transcript *1328A→U*—which has a mutated base in the first of two stem loops (SL1 and SL2a) that comprise the localization signal [2]—undergoes slightly longer runs in the minus-end direction than nonlocalizing transcripts, whereas plus-end run lengths are the same as those of nonlocalizing and wild-type *h* transcripts (Figure 2B). This mode of localization is probably employed by certain endogenous mRNAs. For instance, endogenous *ken* transcripts are only partially enriched apically (Figure S1B), and injected *ken* localizes with kinetics indistinguishable from those of $h^{1328A\rightarrow U}$ (Figures 2A, 2B, and 2D). Conversely, replacing the *h* localization signal with three copies of SL1 (h^{SL1x3}) leads to apical accumulation ~2-fold faster than *h* due to significant increases in the initiation, velocity, and maintenance of minus-end runs (Figures 2A, 2B, and 2D; Table S1). Plus-end motility of h^{SL1x3} is indistinguishable from that of the other transcripts tested (Figure 2B). These findings indicate that mRNA sequences can generate a range of motile behaviors of bidirectional transport complexes.

Egl and BicD Regulate Minus-End-Directed Motility on Microtubules

To investigate how mRNA signals regulate minus-end transport, we first investigated the potential roles of Egalitarian (Egl) and Bicaudal-D (BicD). These proteins are components of a complex required for accumulation of

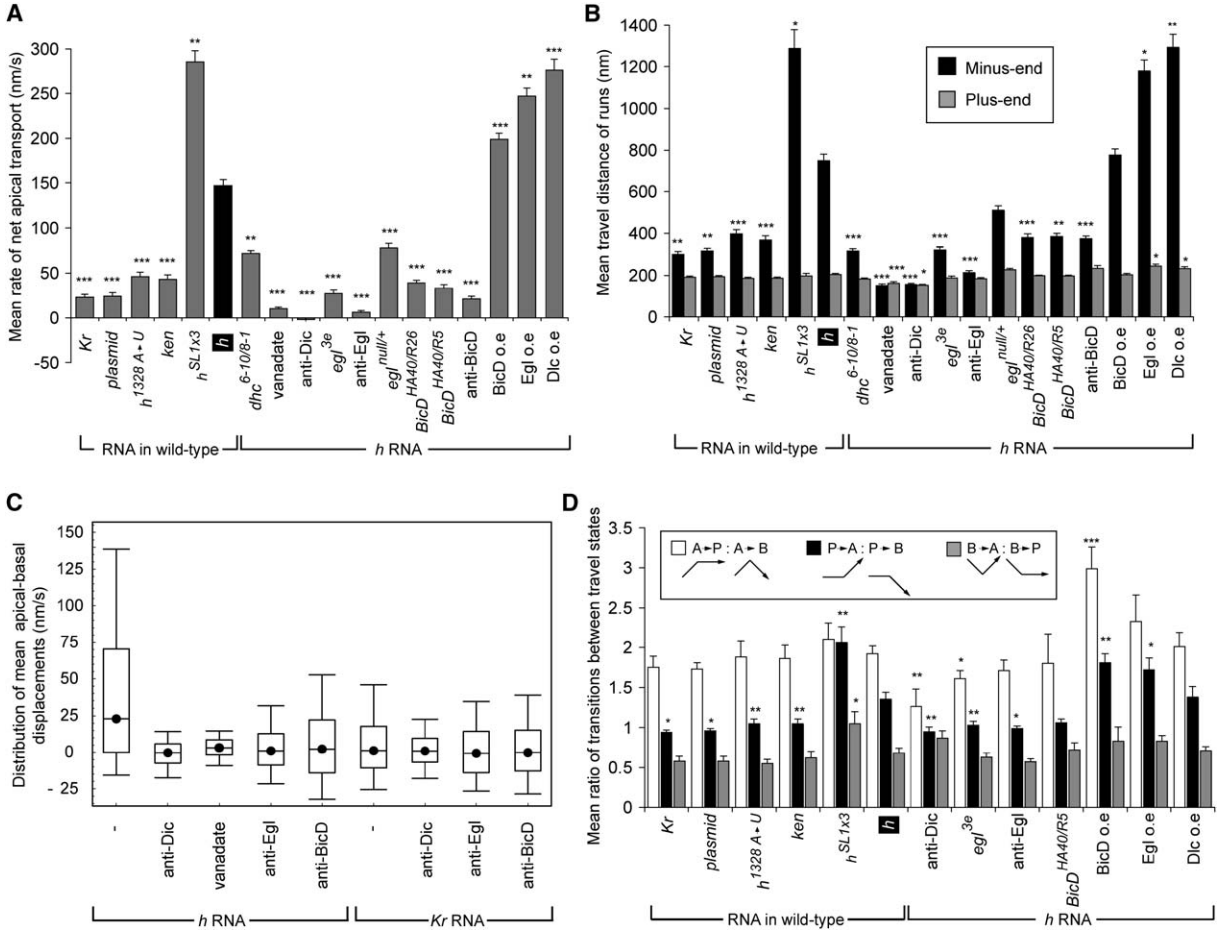

Figure 2. Duration and Frequency of Minus-End-Directed Runs Depends on RNA Signal Architecture, Egl, BicD, and Dynein

(A) Overall rate of apical transport of *h* in wild-type embryos (black bar) compared to other mRNAs in wild-type embryos (to the left) or to *h* upon manipulation of Egl, BicD, or dynein activity (to the right). o.e., overexpression.

(B) Mean travel distance of runs in the minus-end and plus-end direction. Note that inhibition of dynein has a subtle but significant effect on mean plus-end run length.

(C) Distribution of mean apical-basal displacements of particles of *h* or *Kr* with no preinjection (−; similar results were found after injection of control antibodies), or following antibody or vanadate injections. Circle is the median value, and box and whiskers represent 50% and 80% of values, respectively. Negative values represent displacements in the plus-end direction. The most rapid apical-directed movements of *h*, as well as *Kr*, are dependent on Egl and BicD, as well as dynein, leading to a more symmetrical distribution on their inhibition.

(D) Ratios of the occurrence of pauses (P) to plus-end runs (B, basal) following a minus-end run (A, apical) (i.e., A → P: A → B), minus-end runs to plus-end runs following a pause (P → A: P → B), and minus-end runs to pauses following a plus-end run (B → A: B → P). The different transitions are also represented graphically in the key.

*p < 0.05; **p < 0.01; ***p < 0.001 (ANOVA test) compared to *h* in wild-type embryos. Error bars show SEM.

several mRNAs at the minus end of microtubules in *Drosophila* [7–12], and their recruitment to an injected localizing transcript population can be observed above normal cytoplasmic levels [11]. Egl binds directly to Dynein light chain (Dlc) [13], and mammalian BicD associates with components of the dynein and dynactin complexes and recruits membranous vesicles for transport [14–16].

It is not possible to characterize transport in *egl* or *BicD* null embryos because both factors have earlier essential functions. However, hypomorphic mutations in *egl* or *BicD* strongly reduce duration and velocity of minus-end, but not plus-end, runs of both wild-type and $h^{1328A \rightarrow U}$ mutant particles (Figure 2B; Table S1). Weak apical accumulation of endogenous *ken* transcripts is also abolished by these mutations (Figure S1B).

In order to test the full requirements for Egl and BicD in transport of mRNAs, we preinjected embryos with blocking antibodies specific to each protein [11]. We find that antibodies to either protein block net asymmetric movement of mRNAs (Figures 2A and 2C). However, whereas anti-Dic or vanadate injection results in very little movement of mRNAs, anti-Egl- or anti-BicD-injected embryos frequently display short runs of localizing and nonlocalizing mRNAs in both directions (Movie S2; Figure 2C). This limited motility does not result from residual protein activity because it cannot be significantly reduced by injecting the antibodies into partial loss-of-function embryos or by coinjecting the two antibodies (data not shown). Thus, Egl or BicD is not obligatory for linking mRNAs to a motor, which is compatible with the impairment of apical mRNA anchorage upon inhibition of dynein, but not Egl or BicD [17].

Inhibition of Egl and BicD modulates the transitions between pauses, minus-end runs, and plus-end runs

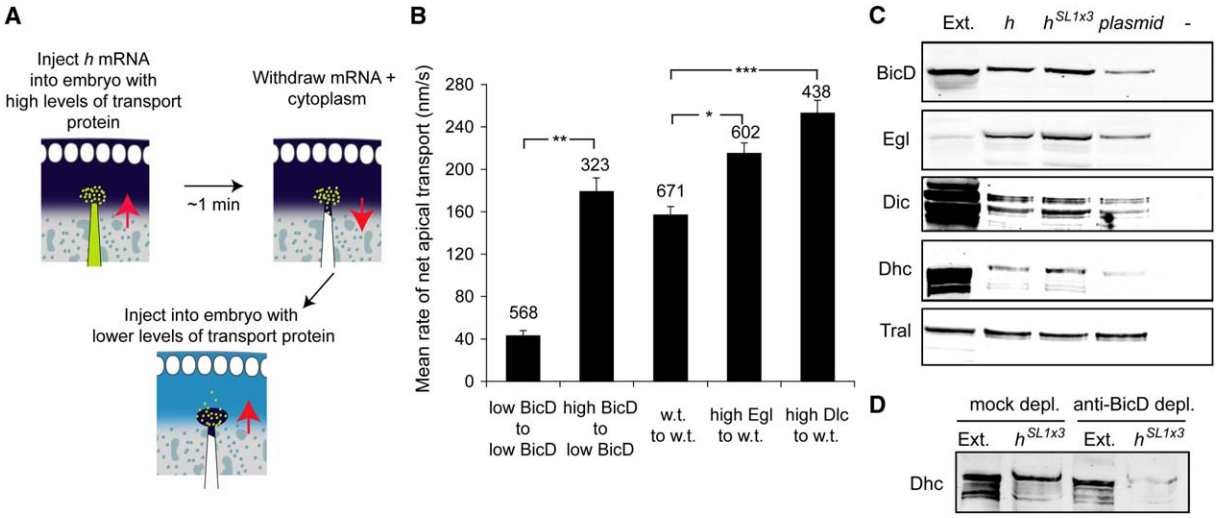

Figure 3. RNA Signals Control Directionality of Movement by Regulating Egl, BicD, and Dynein Recruitment

(A) Scheme for transfer of mRNPs (green dots) between different genotypes.

(B) Rate of apical transport of transferred mRNA particles through the cytoplasm of the acceptor embryo. "High" refers to overexpression; low BicD, $BicD^{HA40/r5}$; w.t., wild-type. Numbers of particle tracks analyzed are shown. Error bars show SEM.

(C) Recruitment of Egl, BicD, and dynein components to mRNAs upon incubation with embryonic extracts. Heterologous transcripts such as those derived from plasmid DNA recruit a small amount of transport proteins (but not other, abundant proteins [data not shown]) consistent with the in vivo requirements for their movement. −, no RNA added to the beads. $h^{1328A \rightarrow U}$ consistently pulls down marginally more of the assayed proteins than plasmid mRNA (data not shown). Probing for Tral protein, which has been implicated in RNA processing [28, 29], reveals similar amounts of each RNA on the beads. By analyzing several experiments, we have determined that the signal for the transport proteins associated with h^{SL1x3} and *plasmid* mRNAs is 1.95 ± 0.39 and 0.50 ± 0.08 of that associated with *h*.

(D) Dhc recruitment to mRNA is reduced, but not abolished, by immunodepletion with anti-BicD antibodies. This treatment reduces BicD levels in the extracts by 95% (data not shown). Ext. is 1/40th of the total cytoplasmic extract used per experiment.

similarly to perturbation of localization signals (Figure 2D). Thus, like RNA localization signals, Egl and BicD promote the initiation and maintenance of rapid minus-end-directed movement of mRNAs along microtubules.

Nonlocalizing transcripts can, however, make use of the Egl/BicD machinery very occasionally (Table S1); a small subset of *Kr* and plasmid mRNA particles undergo relatively long minus-end-directed runs (Movie S3) that are sensitive to inhibition of either protein (Figure 2C).

mRNA Signals Regulate Motility through Numerical Differences in the Transport Proteins They Recruit

The data presented above demonstrate that Egl and BicD augment minus-end-directed movements of mRNAs on microtubules. Interestingly, the frequency, speed, and duration of minus-end runs are significantly reduced when the level of wild-type BicD protein is reduced to ~6% of normal ($BicD^{HA40/r5}$ [9]; Figures 2B and 2D), indicating that BicD has concentration-dependent roles in regulating motility. Indeed, overexpression of BicD results in more efficient apical transport of injected *h* (Figure 2A). Egl and its binding protein Dlc also function dose-dependently; minus-end motility of *h* on microtubules is significantly increased upon overexpression of either protein and reduced by halving *egl* gene dosage (Figures 2A and 2B).

Interestingly, elevating Egl, BicD, or Dlc levels augments minus-end motility in different ways. Very similar to increasing the number of localization elements, overexpression of Egl increases minus-end run length and minus-end velocity and modulates the transitions between travel states (Figures 2B and 2D; Table S1). In contrast, overexpressing BicD only modulates transitions between travel states, and increasing Dlc levels only increases minus-end run length and minus-end velocity (Figures 2B and 2D; Table S1). Egl might therefore function to independently recruit the activities of BicD and Dlc to mRNA signals. Indeed, different domains of Egl mediate association with these two proteins [13].

Consistent with a concentration-dependent role of transport proteins during localization of endogenous mRNAs, there is also a subtle increase in the apical enrichment of endogenous uniform mRNAs upon Egl or Dlc overexpression (Figure 1C). Strikingly, there is only a 2- to 2.5-fold increase in levels of Egl upon its overexpression in all of our experiments (data not shown). Thus, the distinction between net symmetric and asymmetric transcript distribution could reflect subtle differences in the affinities of mRNAs for rather nonselective recognition factors.

Egl, BicD, and Dlc levels could be important for minus-end motility because mRNAs dissociate from them during transit and efficient transport requires reassociation, or because of a function for different numbers of molecules in the transport complex from the outset. To discriminate between these two possibilities, we investigated whether mRNAs assembled in an environment where there is sufficient BicD are sensitive to a subsequent drop in the levels of BicD in the cytoplasm (Figure 3A). This is not the case; *h* transcripts injected into an embryo overexpressing BicD and then withdrawn ~1 min later continue to be transported efficiently through the cytoplasm of $BicD^{HA40/r5}$ acceptor

embryos in which BicD levels are otherwise limiting (Figure 3B).

This finding is associated with neither diffusion of the BicD from the donor embryo following transplantation, confirmed using an epitope tag specific to the overexpressed BicD (data not shown), nor the transplantation procedure, because mRNA transferred either between $BicD^{HA40/r5}$ or between wild-type embryos behaves similarly to when transcripts are simply injected into these genotypes (Figures 2A and 3B). Likewise, the movement of *h* particles exposed to cytoplasm overexpressing Egl or Dlc is not sensitive to the drop in the concentration of these proteins upon transfer to a wild-type embryo (Figure 3B).

These experiments indicate that the only point at which levels of these three proteins is critical is at the initial assembly of transport complexes and suggest that different RNA signals modulate motor activity in a perduring fashion by recruiting different numbers of Egl, BicD, and dynein molecules to each mRNP. Indeed, both Egl and BicD are found complexed with other molecules of themselves in vivo [9, 13]. Higher-order assemblies of dynein also exist in the embryo; measurements of stall forces of minus-end runs of lipid droplets reveal quantized steps of 1.1 pN—equivalent to that of a single motor—up to ~6 pN [18]. Furthermore, the increases in both minus-end run length and velocity we observe for localizing mRNAs and upon augmenting levels of transport proteins are consistent with observations of increasing numbers of active dyneins working together in vitro [19] and in vivo [20, 21].

To directly test whether the extent of minus-end motility of cargoes is associated with the amount of transport proteins nucleated, we injected transcripts of wild-type *h* or the more efficiently localizing construct h^{SL1x3} into embryos and assayed the amount of Egl and BicD assembled on individual mRNA particles in transit. Consistent with such a model, concentration of Egl and BicD above cytoplasmic levels can be observed on many particles of h^{SL1x3} mRNA, but never on particles of *h* (Figure S2A).

Because it is not possible to observe motor components above background levels in these injection experiments, we investigated their recruitment to different RNA signals following incubation with cytoplasmic extracts in vitro. These experiments reveal that localization efficiency of mRNAs does indeed correlate with the amount of dynein components, as well as Egl and BicD, that they assemble (Figure 3C).

Together, our data provide evidence of a novel mechanism in which apical localization signals bias bidirectional motor movement by controlling the number of Egl, BicD, and dynein molecules incorporated into each mRNP (Figure 4). Because short-range bidirectional transport can occur in the absence of RNA localization signals, we envisage that these signals regulate minus-end motility, at least in part, by recruiting dynein motors in addition to those involved in distributing uniform mRNAs. Egl and BicD could function as adaptors that mediate the association of these additional dyneins with localization signals. Consistent with such a role, tethering mammalian BicD sequences to cargoes is sufficient to stimulate dynein recruitment and transport [16], and recruitment of Dhc to localizing mRNAs in vitro is reduced, but not abolished, upon immunodepletion of BicD from extracts (Figure 3D).

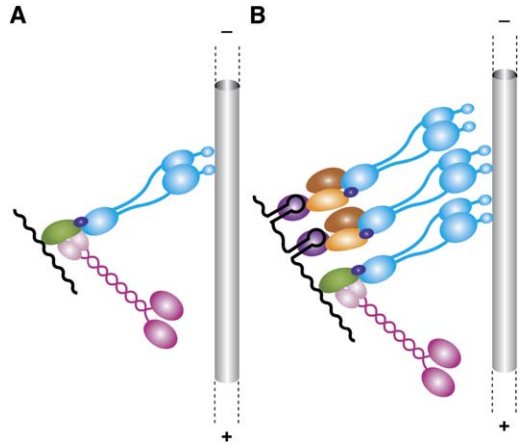

Figure 4. Model

Transcripts (black) are recognized by a factor(s) with general affinity for mRNA (green), which links them to a bidirectional motor complex consisting of dynein (blue) and an unidentified plus-end-directed motor activity (magenta). In the absence of RNA localization elements (A), this machinery facilitates exploration of the cytoplasm by nonlocalizing mRNAs. In the presence of RNA signals (stem-loop structures) (B), net transport is achieved by their recruitment of additional copies of the dynein complex, which increases the probability of persistent minus-end-directed transport on microtubules (gray). Nucleation of additional dyneins is mediated by an unknown RNA recognition factor(s) (purple), Egl (yellow), and BicD (orange). RNA signals can generate different classes of motion through differences in the affinity or number of binding sites for this complex. For simplicity, multiple motors are shown attached to a single transcript, although interactions could also be spread over multiple mRNA molecules within a particle. Precise molecular interactions within the complex are speculative, although Egl is known to bind Dlc (dark blue), and BicD associates with components of the dynein complex and the accessory complex dynactin (not shown here).

Nonetheless, our finding that increasing levels of Egl, BicD, or Dlc can modulate transport argues against a strict linear pathway of assembly. One intriguing explanation, which could also account for the substantial differences in the relative amounts of Egl and BicD assembled on individual mRNA particles (Figure S2A), is that not all of the binding sites within the RNA:motor assembly must be saturated before transport is initiated. Modulating the number of mRNA elements or the concentration of each of the transport proteins could therefore alter the average number of fully functional Egl/BicD/dynein complexes assembled on each mRNP. Such a probabilistic mechanism could also account for the large variation in motile behaviors exhibited by particles of the same mRNA (Figure S2B; Movie S1).

Although differential motor recruitment appears to be one important mechanism to generate different classes of mRNA motion, our data hint at the existence of additional regulatory processes. The anti-BicD antibody largely uncouples minus-end run lengths from velocity (Table S1), and overexpression of BicD alters transitions between travel states, but not run lengths or velocity. Thus, BicD is likely to have additional roles in regulating dynein activity. This could be through its binding to the dynactin complex [14, 15], which is likely to play a key role in coordinating minus- and plus-end-directed motor activity [4, 22]. Indeed, Egl and BicD levels could

regulate the hypothesized switch mechanism that is proposed to coordinate opposite polarity motor activities and determine when runs end [23, 24].

Perspective

In many cell types, mRNAs exhibit net transport toward the plus ends of microtubules [25, 26]. These distributions could result from modulation of a related bidirectional transport complex using mRNA elements that preferentially nucleate or stimulate plus-end-directed motor activity [27]. Our findings also suggest that different organelles, vesicles, and macromolecules could assume a wide range of polarized distributions within the same cell by balancing opposite polarity motor activities through numerical differences in the same repertoire of transport proteins.

Experimental Procedures

Detailed experimental procedures are included in the Supplemental Data.

Supplemental Data

The Supplemental Data include Experimental Procedures, two figures, one table, and three movies and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/14/1447/DC1/.

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