Centrosomal Enrichment and Proteasomal Degradation of SYS-1/ β -catenin Requires the Microtubule Motor Dynein

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Abstract

The *C. elegans* Wnt/ β -catenin Asymmetry (W β A) pathway utilizes asymmetric regulation of SYS-1/ β -catenin and POP-1/TCF coactivators. W β A differentially regulates gene expression during cell fate decisions, specifically by asymmetric localization of determinants in mother cells to produce daughters biased towards their appropriate cell fate. Despite the induction of asymmetry, β -catenin localizes symmetrically to mitotic centrosomes in both mammals and *C. elegans*. Due to the mitosis-specific localization of SYS-1 to centrosomes and enrichment of SYS-1 at kinetochore microtubules when SYS-1 centrosomal loading is disrupted, we investigated active trafficking in SYS-1 centrosomal localization. Here, we demonstrate that trafficking by microtubule motor dynein is required to maintain SYS-1 centrosomal enrichment, by dynein RNAi-mediated decreases in SYS-1 centrosomal enrichment and by temperature-sensitive allele of the dynein heavy chain. Conversely, we observe depletion of microtubules by Nocodazole treatment or RNAi of dynein-proteasome adapter ECPS-1 exhibits increased centrosomal enrichment of SYS-1. Moreover, disruptions to SYS-1 or negative regulator microtubule trafficking are sufficient to significantly exacerbate SYS-1 dependent cell fate misspecifications. We propose a model whereby retrograde microtubule-mediated trafficking enables SYS-1 enrichment at centrosomes, enhancing its eventual proteasomal degradation. These studies support the link between centrosomal localization and enhancement of proteasomal degradation, particularly for proteins not generally considered 'centrosomal'.

Keywords

Development, Wnt, β -catenin, microtubule, ECM29, dynein, proteasome, trafficking, elegans, asymmetry, cell cycle, robustness

Introduction

During metazoan development, cells must robustly respond to and integrate multiple signaling pathways to appropriately specify and populate tissues. This is of further importance in the developing embryo, where cell division rapidly alternates between S-phase and mitosis while simultaneously specifying tissues and entire germ layers (Pintard and Bowerman 2019). As such, the cell's response to these signals and the subsequent establishment of asymmetry often begins before asymmetric daughter cells are even 'born' via an asymmetric cell division (ACD) (Sunchu and Cabernard 2020; Juanes, 2020; Fuchs and Chen, 2013; Gómez-López, Petrisch et al 2014). Among the signaling pathways utilized in development is Wnt/ β -catenin signaling, wherein the binding of Wnt ligands disrupts β -catenin degradation via dismantling an intracellular destruction complex. This effectively stabilizes β -catenin, enabling it to activate target genes with transcriptional activator TCF/LEF (Nusse and Clevers 2017). Wnt/ β -catenin signaling is specialized in its ability to regulate both polarity and proliferation – enabling developing organisms to orient and expand tissues simultaneously for appropriate size and morphology (Loh et al 2016). Due to its role in both proliferation and polarity, Wnt/ β -catenin signaling is readily adaptable to the adult organism throughout development.

Wnt/ β -catenin is particularly well adapted to the organization of a patterned stem cell niche for proliferative tissue maintenance (Pinto, Clevers et al 2003; Reilein, Kalderon et al 2017). However, this function in adult tissue homeostasis also associates deleterious dysregulation of Wnt/ β -catenin signaling with disease states. Throughout transduction of the Wnt signal are proteins vulnerable to oncogenic mutation (Yang, yen 2011; Zhunussova, Djansugurova et al 2019; Liu, Thibodeau et al 2000). However, these often affect their tumorigenicity via dysregulation of the pathway effector β -catenin, which can promote proliferation via over- and mis-expression of targets like Cyclin D1 and cMyc (Niehrs, Acebron 2012; He, Kinzler et al 1998; Shtutman, Ben-Ze'ev et al 1999; Tetsu and McCormick, 1999). Moreover, β -

catenin is a bifunctional protein in mammalian systems. That is, it is distributed between the previously described signaling role while also maintaining cell-cell adhesion at adherens junctions (Niehrs, Acebron 2012; Reya, Clevers 2005), where it facilitates the interaction between membrane protein E-cadherin and the cytoskeleton. The combination of these roles for β -catenin makes it an attractive target for studying tumorigenesis, both due to hyperproliferative phenotypes and ease of metastasis. However, the dual functions can also lead to confounding pleiotropic phenotypes after manipulation of β -catenin activity (Reya, Clevers 2005; Tejpar, Alman et al 1999; Geyer, Reis-Filho et al 2011).

Unlike β -catenin in the canonically described Wnt signaling pathway, *C. elegans* has distributed the adhesive and signaling roles across its four β -catenins (Sawa, 2012). Here we focus specifically on the *C. elegans* β -catenin SYS-1 for its role as a transcriptional co-activator in a modified Wnt/ β -cat signaling pathway, the Wnt/ β -catenin Asymmetry pathway(W β A) (Phillips, Kimble et al 2007). The W β A pathway is a specialized application of the more basic Wnt signaling paradigm, wherein regulation of the co-activator SYS-1 (β -catenin) modulates the activity of transcription factor POP-1 (TCF), during asymmetric cell division (Baldwin and Phillips 2018, Lam and Phillips 2017). Mother cells undergoing a W β A-driven division polarize components of the Wnt signaling pathway along the axis of the division – distributing positive regulators of a Wnt response, the Frizzled receptor and downstream effector Disheveled, to the signaled pole of the cell, and negative regulators, members of the β -Catenin destruction complex APC and Axin, to the unsignaled pole (Baldwin and Phillips 2014; Baldwin, Phillips et al 2016; Sugioka, Sawa et al 2011; Mizumoto and Sawa, 2007). Daughter cells in such a division therefore inherit Wnt signaling components that bias them toward their appropriate cell fate. Contrary to its known role asymmetrically activating Wnt target genes, SYS-1 (and indeed, mammalian β -catenin (Mbom, Barth et al 2014; Vora, Phillips et al 2020)), unexpectedly localizes symmetrically to mother cell mitotic centrosomes (Phillips, Kimble et al 2007; Huang, Lin et al 2007; Vora and Phillips 2015).

Previous work has demonstrated that mother cell centrosomal localization of SYS-1 limits SYS-1 levels in daughter cells in a proteasome dependent manner (Vora and Phillips 2015). Differential localization of the proteasome during the cell cycle (Amsterdam, Baumeister et al 1993), as well as interactor-specific localization of the proteasome for intracellular structuring (Baldin, Cux et al 2008), suggest that proteasome regulation, localization, and activity are interdependent. Proteasomal subunits have specifically been shown to co-sediment with centrosomal proteins and perinuclear inclusions, indicative of centrosomal localization (Wigley, Thomas et al 1999; Wojcik, DeMartino 2003). Similarly, turnover of the SYS-1 mobile fraction at mitotic centrosomes is enhanced by RPT-4 proteasomal activity (Vora and Phillips 2015). When the localization of SYS-1 to centrosomes is reduced by *rsa-2(RNAi)*, resultant daughter cells retain 40% additional SYS-1 protein. Perhaps, then, the centrosome is a region where SYS-1 regulatory interactions are concentrated to coordinate its retention and effect on cell fate during mitosis.

The directionality and temporal specificity of SYS-1 enrichment at only mitotic centrosomes implied that a more active localization mechanism might be at work, as compared to a passive diffusion-capture mechanism. SYS-1 motility is most directly demonstrated in that the cell maintains a proteasome-dependent, 100% mobile population of centrosomal SYS-1, which is recycled within 120 seconds (Vora and Phillips 2015). Cells appear to maintain robust localization of SYS-1 to the mitotic centrosome, while the interphase centrosome remains undetectable via SYS-1 fluorescence (Vora and Phillips 2015). However, the mechanism of such temporally specific SYS-1 centrosomal localization is unknown. Several observations indicate that cells utilize the microtubule cytoskeletal network for rapid and directional regulation of specific cargo to precise locales, including: that dynein conditionally traffics transcription factors due to neuronal injury or signaling status, (Ben-Yaakov, Fainzilber et al 2012; Mikenberg, Kaltschmidt et al 2007) and that dynein more generally can enrich endocytic recycling components around the Microtubule Organizing Center (MTOC) (Horgan, Mccaffrey et al 2011) or be

sufficient to prevent pathogenic accumulation of RNA binding proteins (Deshiumar, Tsuboi et al 2021). Therefore, we hypothesized that the role of the mitotic centrosome as an MTOC could facilitate the rapid transport of SYS-1 to the pericentriolar material specifically in dividing cells.

The rapid and cell cycle phase-specific enrichment of SYS-1 at the centrosome suggests that it may also depend on active transport. Further, accumulation at a highly active MTOC like the mitotic centrosome is most likely accomplished by the microtubule minus end-directed dynein motor complex (Vora and Phillips 2015; Priyanga, Bhaka-Guha et al 2021). The dynein motor is a multi-subunit complex composed of a pair of ATP-ase, microtubule-binding heavy chains responsible for the force-generating steps of the motor, and a variable complement of smaller light, intermediate, and accessory chains that alter the cargo binding or processivity of the motor (Reck-Peterson, Carter et al 2018; Jha and Surrey 2015). The dynein motor is multifunctional - it provides pulling forces, positions organelles, and can mediate rapid and conditional movement of proteins and RNAs (Carminati and Stearns 1997; Woźniak, Allan et al 2009; Harada, Hirokawa et al 1998; Hanz, Fainzilber et al 2003; Bullock and Ish-Horowicz 2001). For example, injured rat axons have demonstrated a dynein intermediate chain-dependent nuclear localization and coimmunoprecipitation of the transcription factor STAT3, in order to appropriately adopt an injury-dependent transcriptional response (Ben-Yaakov, Fainzilber et al 2012). Dynein specifically is the motor complex most often directed towards the MTOC and is known to be active during mitosis (Jha and Surrey 2015). Therefore, we investigated dynein-dependent trafficking as an ideal mechanism for SYS-1 localization to the MTOC mitotic centrosomes.

Here we demonstrate that microtubules and minus-end microtubule-mediated trafficking are required for the centrosomal recruitment of both SYS-1 and the negative regulators responsible for its regulation there. We evaluate the extent to which SYS-1 utilizes dynein-mediated trafficking to accumulate at centrosomes. Using both long- and short-term disruptions to dynein and microtubule-mediated trafficking to evaluate SYS-1 localization and SYS-1-dependent cellular activity up to and

including cell fate changes, we demonstrate that SYS-1 and its negative regulators both require microtubule-mediated trafficking for appropriate regulation of SYS-1. Together, these data characterize a system for enhancing the robustness of the W β A signaling pathway by maintaining consistent interaction of SYS-1 with negative regulators at the mitotic centrosome.

Results

SYS-1 Overexpression and Centrosomal Uncoupling Increases SYS-1 Localization to Regions of Dynein Enrichment

To test the role of microtubule-mediated trafficking in SYS-1 centrosomal localization, we investigated what trafficking mechanisms are necessary for SYS-1 regulation. We compared fluorescently tagged SYS-1 protein localization to that of MT trafficking components. Of most interest was the cytoplasmic dynein motor complex, since its components have been shown to localize to mitotic centrosomes, kinetochore microtubules, and cortically across species (Schmidt, Van den Heuvel et al 2017; Tame, Medema et al 2014). The dynein localization pattern corresponded to that of GFP::SYS-1 to cortical and microtubule dense regions. Moreover, kinetochore and cortical localization was more common upon centrosomal uncoupling, complete loss of visible enrichment of centrosomal SYS-1, by RNAi depletion of the spindle assembly and scaffolding protein RSA-2(Fig. 1A).

While GFP::SYS-1 has been observed at mitotic centrosomes in many dividing cells throughout development, localization to these cortical and kinetochore 'secondary' sites is uncommon (accumulation at either or both of these sites occurring in 34.5% of embryos) in untreated embryos but the frequency of this localization approximately doubles, to 69.1%, with *rsa-2(RNAi)* (Fig.1A). Uncoupling SYS-1 protein from the centrosome by *rsa-2(RNAi)*, thereby decreasing processing of the protein, allows us to more frequently observe SYS-1 at sites of dynein localization (Vora and Phillips 2015, Vora and Phillips 2017)(Fig.1A). Localization to kinetochore microtubules, cortex, and the MTOC centrosome

therefore suggests that SYS-1 may be a cargo of the comparably localized dynein and kinesin microtubule motors (Ferenz, Wadsworth et al 2010; Schmidt, Van den Heuvel et al 2017).

Knockdown of Dynein Complex Subunits Reduces SYS-1 Centrosomal Localization

Given the observation that SYS-1 localizes to cortical and kinetochore sites that also exhibit enrichment for microtubule motors, we investigated a functional role for microtubule-mediated trafficking in SYS-1 localization. We began by performing an RNAi screen against various components of the dynein motor complex and associated proteins, eventually expanding outward to additional motors and microtubule-affecting proteins (Fig. S1). Specifically, by RNAi depletion of these microtubule trafficking proteins, we assayed the centrosomal enrichment of GFP::SYS-1. We expected depletion of transport proteins that play a role in SYS-1 centrosomal transport to be defective in the maintenance of SYS-1 centrosomal enrichment when SYS-1 centrosomal clearance was otherwise unaffected. In order to evaluate the centrosome-specific enrichment of SYS-1, we considered two ways of comparing the extent of fluorescently-tagged SYS-1 localization to centrosomes: 1) the centrosomal enrichment index (CEI)(similar to that described in Gao, Smith et al 2017), the centrosomal fluorescence intensity adjusted by subtraction of the background cytoplasmic fluorescent intensity of the surrounding cell, to control for backgrounds that may pleiotropically influence SYS-1 expression, and 2) the SYS-1 CEI evaluated as a ratio to the same enrichment factor of representative Peri Centriolar Material (PCM) factor γ -tubulin (Tubulin Enrichment Index, or TEI) , to avoid false positives via defects in PCM maturation (CEI/TEI) (Fig. 1B-B''). We then compared these centrosomal enrichment metrics to those seen in established *rsa-2(RNAi)* centrosomal uncoupling.

As expected, depletion of several dynein subunit knockdowns presented pleiotropic and deleterious effects, including oogenesis and early patterning defects which precluded our SYS-1

localization assay. We therefore treated animals with partial RNAi knockdowns by commencing RNAi feeding 12-24 hours before imaging as necessary, based on the phenotypic severity. While no treatment disrupted SYS-1 centrosomal levels as severely as *rsa-2(RNAi)*, we did observe several statistically significant decreases in centrosomal SYS-1 enrichment (Fig. 2C – C', Fig. S1). Furthermore, while the total population of some treatments displayed only an intermediate mean loss of SYS-1 enrichment, the distribution of SYS-1 CEI values observed in dynein subunit knockdowns extends from wild-type to an *rsa-2(RNAi)*-like disruption (e.g., Fig. 2 Supplement 1). Dynein knockdown-induced decreases in SYS-1 CEI or CEI/TEI are masked somewhat in the population average by their broad range in phenotype and/or RNAi penetrance (Fig. 1C-C', Fig S2). Most notably, *dlc-1(RNAi)* and *dylt-1(RNAi)* each exhibit a significant reduction in centrosomal SYS-1 localization disproportionately larger than the effect on γ -Tubulin. Loss of centrosomal SYS-1 enrichment was specifically more visible in a transgene driven by the endogenous SYS-1 promoter ($P_{\text{sys-1}}::\text{VENUS}::\text{SYS-1}$), as compared to SYS-1 driven by the stronger *pie-1* promoter ($P_{\text{pie-1}}::\text{GFP}::\text{SYS-1}$) (Fig 2C-C').

While we observed dynein-dependent decreases in SYS-1 localization, this could be because of changes in the RSA-2 centrosomal scaffold, which is responsible for capturing SYS-1 at mitotic centrosomes (Vora and Phillips 2015, Schlaitz, Hyman et al 2007). While some dynein subunits did show slight decreases in centrosomal GFP::RSA-2 – 16% reduction in *dlc-1(RNAi)* embryos and 14% in *dylt-1(RNAi)* embryos. Embryos depleted of DLC-1 by RNAi exhibit double this effect on SYS-1 localization, with 39% and 42% reductions in $P_{\text{sys-1}}::\text{VENUS}::\text{SYS-1}$ CEI and CEI/TEI, respectively. DYLT-1 depletion exhibited a similar 14% decrease in CEI alone, but a 39% decrease in CEI/TEI. Both knockdowns suggest that tagged SYS-1 is disproportionately removed from centrosomes in dynein knockdowns (Fig. S3)

These data suggest that dynein does have a role in the localization of SYS-1 to centrosomes, but redundancy between components of the dynein motor or between the dynein motor and other processes may limit the effect any one subunit has on SYS-1 localization. Alternately, while mutants are

often severely deleterious, variability of RNAi efficacy may have resulted in incomplete depletion of SYS-1 relevant dynein subunits. Similarly, the pleiotropic effects of dynein depletion may also have superseded effects on SYS-1 localization, leading to the evaluation of only partial knockdowns. In order to address these issues, we investigated the role of the dynein motor in SYS-1 localization at a more precise temporal resolution via conditional dynein disruption.

Temporally Restricted Dynein Loss of Function Reveals Dynein Requirement for Centrosomal SYS-1 Accumulation.

While our screen showed that dynein knockdown results in significant reduction of centrosomal SYS-1, with rare individuals exhibiting *rsa-2(RNAi)*-like loss of centrosomal SYS-1 accumulation, we were unable to completely recreate a population exhibiting consistently severe disruption to SYS-1 CEI. To distinguish the variety in effect on SYS-1 localization effects between functional redundancies limiting the effects of dynein depletion and the confounding deleterious effects described above, we utilized a temperature sensitive allele of the cytoplasmic dynein heavy chain, *dhc-1(or195ts)*, to conditionally inactivate the cytoplasmic dynein heavy chain. This allele, identified in the Bowerman lab, introduces a lesion to the microtubule binding stalk of DHC-1. This lesion renders the motor slightly hypomorphic at the permissive 15°C (with 91% of embryos remaining viable and fertile) but undergo complete mitotic spindle collapse as little as 30 seconds upon upshift, suggesting that DHC-1 motor function has been nearly completely ablated (O’rourke, Bowerman et al. 2007).

Therefore, we expected that combining the *dhc-1(ts)* allele with fluorescently tagged SYS-1 would allow us to examine a more penetrant dynein knockdown. Notably, this allowed us to overcome both redundancy between dynein accessory chains in SYS-1 trafficking as well as pleiotropic effects of dynein depletion that preclude the observation of mitotic centrosomal SYS-1 (Fig. 2A). As described above, we compared the CEI of $P_{\text{SYS-1}}::\text{VENUS}::\text{SYS-1}$ (VENUS::SYS-1) to that of mCherry:: γ -Tubulin (TEI).

SYS-1 and γ -Tubulin are both recruited to centrosomes at a much higher rate during mitosis than in interphase (Vora and Phillips 2015; Raynaud-Messina and Merdes 2007). Because *dhc-1(ts)* limits the extent of γ -Tubulin recruitment (Fig. 2B-C), evaluating SYS-1 CEI as a ratio to γ -Tubulin TEI ensures that decreases to the SYS-1 CEI must be disproportionately large to be numerically distinct. Consistent with the published hypomorphic nature of the allele at the permissive temperature (O’rourke, Bowerman et al 2007), imaging *dhc-1(ts)* embryos, shows notable decrease in the SYS-1 CEI of embryos kept at the permissive temperature (Fig. 2 B-D). Following 30-45 seconds of temperature upshift, the SYS-1 localization hypomorphy is enhanced, severely reducing SYS-1 centrosomal enrichment (Fig. 2B-C). While there was a decrease in centrosome size, presumably due to roles for dynein in centrosome maturation (Priyanga, Bhakta-Guha et al 2021), we accounted for this by assessing SYS-1 CEI as a ratio to the enrichment of γ -Tubulin TEI. We observed a CEI/TEI decrease corresponding to decreased centrosomal SYS-1 accumulation, proportionally greater than the reduction in γ -tubulin (Fig. 2D’). Combined with the individual dynein subunit knockdown, the more penetrant phenotype of *dhc-1(ts)* mutants indicates that SYS-1 requires the dynein microtubule motor complex to localize appropriately to centrosomes. Additionally, it seems likely that redundancy of peripheral motor components or incomplete knockdown is responsible for the variability within individual subunit depletions seen above (Fig 1C-C’).

MT depolymerization experiments reveal that SYS-1 centrosomal dynamics are affected both positively and negatively by Microtubule-Mediated trafficking

To determine the extent to which microtubule-based trafficking in general is responsible for SYS-1 centrosomal localization, we treated gravid adults and their embryos with >100 μ M Nocodazole, a microtubule-destabilizing agent. This dose, while sufficient for a rapid acute response in adult animals, either caused no effect on fertilized embryos because of the eggshell or prevented the production of

additional embryos via germline arrest (data not shown). We therefore turned to a lower dose, longer-term Nocodazole treatment: 12 hours at 25 μ g/mL (83 μ M). Maternal Nocodazole dosing allowed us to obtain embryos with limited microtubule networks, but sufficient spindle development to enter mitosis (Fig. 3A). Surprisingly, when treated with nocodazole, the centrosomal enrichment of the brighter P_{pie-1}::GFP::SYS-1 (GFP::SYS-1) increased after Nocodazole treatment (Fig. 3B, C).

The observed SYS-1 CEI increase is similar to the phenotype observed in RPT-4 depletion, which decreases proteasome function (Vora and Phillips, 2015). To ensure that this change in SYS-1 localization was not because of off-target effects, we compared maternal feeding to that of permeabilized embryos. At 10.5 μ M these embryos formed sufficient microtubule networks to enter mitosis and form spindles, but these spindles were often inappropriately rotated or positioned (61% of imaged embryos, n=11/18), suggesting that astral microtubules have been severely disrupted (Fig. S4A). We observed a similar CEI increase in GFP::SYS-1 in these embryos (Fig. S4B). This is consistent with a model where both SYS-1 recruitment to centrosomes and SYS-1 proteasomal degradation are affected by disruptions to microtubule mediated trafficking. Though microtubule poisoning by Nocodazole and disruption of dynein function have opposite effects on SYS-1 centrosomal enrichment, either change in SYS-1 centrosomal enrichment may be indicative of a mis-localized regulatory process.

As different subsets of dynein and microtubule depletions exhibit either increased or decreased SYS-1 CEI, we investigated a combination of these treatments to observe the behavior of SYS-1 upon further disruption to microtubule-mediated trafficking. Moreover, while both temperature upshift for *dhc-1(ts)* and Nocodazole treatments significantly affect SYS-1 centrosomal localization, neither treatment appears to completely disrupt microtubule mediated trafficking. Nocodazole-treated embryos still exhibit some kinetochore microtubules (Fig. 3A) but can tolerate weakly poisoned microtubules for some time. Conversely, *dhc-1(ts)* embryos demonstrate such severe loss of dynein function at the

restrictive temperature that short-term temperature upshifts are required to establish spindles and maintain viability, suggesting some portion of centrosomal trafficking during mitosis still occurs. We therefore tested the combination of these treatments.

In contrast to embryos with GFP::SYS-1 driven by the strong early embryonic PIE-1 promoter (Fig. 3 B-C), embryos containing $P_{\text{sys-1}}::\text{VENUS}::\text{SYS-1}$, driven by the weaker endogenous SYS-1 promoter, exhibit only a slight, non-significant, increase in CEI/TEI when treated with nocodazole (Fig. 3D, 1st and 2nd columns). However, when $P_{\text{sys-1}}::\text{VENUS}::\text{SYS-1}$ centrosomal enrichment is challenged by the hypomorphic *dhc-1(ts)* at the permissive temperature, nocodazole treatment is sufficient to increase SYS-1 centrosomal recruitment. The finding that sensitizing embryos for dynein trafficking defects is required to observe a nocodazole phenotype for $P_{\text{sys-1}}::\text{VENUS}::\text{SYS-1}$, but not for $P_{\text{pie-1}}::\text{GFP}::\text{SYS-1}$ indicates that the difference in transgene expression uncover a differential nocodazole sensitivity in centrosomal recruitment of SYS-1. However, nocodazole-mediated increase in $P_{\text{pie-1}}::\text{GFP}::\text{SYS-1}$ centrosomal enrichment is no longer observed when the nocodazole treated *dhc-1(ts)* embryos are upshifted to the restrictive temperature, suggesting that the increased SYS-1 centrosomal enrichment seen in nocodazole treated embryos still requires dynein trafficking.

These observations describe a centrosomal relationship wherein treatments limiting the coverage of microtubule-mediated dynein trafficking (i.e. nocodazole treatment) increase centrosomal SYS-1 because of relatively inefficient or rare trafficking events for SYS-1 negative regulators, while transient severe disruptions to dynein trafficking processivity (dynein subunit RNAi depletion, *dhc-1(ts)* temperature upshift, upshift + nocodazole) reveal that SYS-1 centrosomal enrichment requires continual replenishment via a functional microtubule motor.

If the nocodazole-induced SYS-1 CEI increase described above is indeed because of a role for microtubule mediated trafficking in the centrosomal enrichment of a component of the SYS-1

degradation pathway, we should expect proteolytic activity to be similarly mis-localized upon trafficking disruption. Therefore, we decided to test the idea that proteasomal trafficking to, and enrichment at, the centrosome was also perturbed in nocodazole-affected microtubule networks. We first attempted to localize the proteasome. While the centrosome has been demonstrated to exhibit proteolytic activity (Vora and Phillips 2015; Vora and Phillips 2016; Hames, Fry et al 2005; Peel, O'Connell et al 2012; Wigley, Thomas et al 1999; Fabunmi, DeMartino et al 2000), existing immunohistochemical reagents for mammalian proteasome subunits did not display any distinct localization pattern. An mCherry tag on proteasome subunit RPT-1 did demonstrate a kinetochore microtubule enrichment like that of both DHC-1 and SYS-1 but appeared absent from centrosomes. Inability to localize tagged RPT-1 may be due to a relatively low RPT-1 copy number per proteasome, or correspondingly low proteasome number, limiting its relative enrichment. Alternatively, fluorescently tagged proteins have shown evidence of degradation of the tag at a site of active proteolysis, particularly if such a tag limits proteasome activity (Huang, Lobel et al 2014). To circumvent this issue, we assayed for centrosomal proteasome activity indirectly by functionally testing an additional centrosomal target of the RPT-4 containing proteasome, the centriole duplicating kinase ZYG-1 (O'Connell, White et al 2001; Peel, O'Connell et al 2012). In addition, we assayed the centrosomal role of the *C. elegans* homolog of mammalian ECM29, ECPS-1, which is an adaptor protein that enhances local proteolytic activity of proteasomes via its function linking the dynein motor complex and the proteasome and regulating 26S proteasome distribution (Gorbea, Rechsteiner et al 2004; Gorbea, Rechsteiner et al 2010; Hsu, Cheng et al 2015; Ibañez-Vega, Yuseff et al 2021).

Knockdown of ECM29 homolog ECPS-1 (formerly D2045.2) resulted in embryos that displayed an average 33.4% increase in centrosomal P_{pie-1}::GFP::SYS-1 centrosomal enrichment, similar to both Nocodazole-treated and *rpt-4(RNAi)* embryos (Fig. 4A, Fig. 3A-C). Therefore, the overall increase in SYS-1 CEI seen in nocodazole and *ecps-1(RNAi)* treated embryos suggests that microtubule mediated

trafficking enhances the removal of SYS-1 from centrosomes as well. Given the role of ECM29 in proteasomal distribution via microtubule-mediated trafficking rather than directly in protein degradation, these data suggest that an improperly localized proteasome in *ecps-1(RNAi)* results in dysregulation of SYS-1 similar to that seen in proteasome functional knockdown (*rpt-4(RNAi)*) or broader depletion of microtubules (Nocodazole treatment). To determine if ECPS-1 knockdown is specific to SYS-1 dysregulation, or perhaps plays a more general role in protein regulation, we assayed an additional target. The ZYG-1 protein, a regulatory kinase controlling centriolar duplication, is both centrosomally enriched during mitosis and negatively regulated by the RPT-4 proteasome (Peel, O'Connell et al 2012). Utilizing a fluorescently tagged ZYG-1 fragment demonstrated to mirror the localization and dynamics of the endogenous protein (Peters, O'Connell et al 2010), we demonstrated 20% and 27% increased accumulation of ZYG-1 in both proteasome knockdown *rpt-4(RNAi)* and *ecps-1(RNAi)* treatments, respectively. The similarity of phenotype between Nocodazole, ECPS-1 depletion, and RPT-4 proteasome knockdown implies a microtubule-mediated trafficking link between centrosomally localized proteins and their eventual degradation by the RPT-4 proteasome.

Since SYS-1 centrosomal recruitment depends on rapid degradation via the centrosomal proteasome (Vora and Phillips 2015), we assayed the turnover rate of centrosomal SYS-1 in *ecps-1(RNAi)* by FRAP. Assaying SYS-1 centrosomal turnover allowed us to assess the motility of centrosomal SYS-1. Comparison of GFP::SYS-1 recovery after photobleach demonstrates that depletion of ECPS exhibits an intermediate phenotype between proteasome depletion via *rpt-4(RNAi)* and negative controls (5G-G"). While the initial rate of *ecps-1(RNAi)* GFP::SYS-1 recovery was similar to that seen in wild type embryos, the late recovery phase of *ecps-1(RNAi)* embryos showed an early SYS-1 recovery plateau, evidence of a significantly reduced mobile fraction intermediate to wild type and that of proteasome depleted *rpt-4(RNAi)* embryos (Fig. 4E,G). The recovery curve observed in *ecps-1(RNAi)* suggests that SYS-1 recovers at an approximately wild type rate in *ecps-1(RNAi)* embryos but remains approximately as immobile as

in proteasome-depleted embryos. Therefore, ECPS-1 depleted embryos appear to degrade SYS-1 incompletely, as observed in the RPT-4 proteasome depletion, but with sufficient protein turnover to enable wild type recruitment of newly trafficked SYS-1.

While both $P_{pie-1}::SYS-1::GFP$ and $P_{pie-1}::GFP::ZYG-1c$ exhibit an increase in their CEI because of ECPS-1 depletion, their turnover rates at the centrosome are not similarly affected. The differential motility between SYS-1 and ZYG-1c may be due to diverging regulatory pathways en route to the RPT-4 proteasome, or differential sink volumes at the mitotic centrosome. Meanwhile, ECPS-1 depleted embryos may be unaffected in protein clearance per proteasome, but the delayed or partial localization of the centrosomal proteasome could result in hierarchical stabilization of a spatially or temporally inaccessible fraction consisting of highly expressed and continually trafficked proteins, like SYS-1.

Dynein Trafficking is Required for Restriction of Wnt-Signaled Cell Fates

Having established a two-fold role for SYS-1 regulation in microtubule-mediated trafficking, we investigated the role microtubule trafficking played in regulating SYS-1 dependent cell fate decisions. Since the SYS-1 centrosomal localization pattern has been seen across all examined SYS-1-expressing tissues (Phillips, Kimble et al 2007; Huang, Lin et al 2007; Baldwin and Phillips 2018; Lam and Phillips 2017), we investigated cell fate changes in embryonic and larval tissues across one embryonic and two larval SYS-1 dependent cell fate decisions. These included the transcriptional reporter of the $W\beta A$ target gene, *end-1*, in the establishment of SYS-1 dependent endoderm, and an anatomical marker for larval specification of the stem cell-like cells of the hypodermal seam and the germline stem cell niche. First, to directly assay the effect of centrosomal dysregulation of transcriptional activator SYS-1, we investigated the nuclear enrichment of $GFP::SYS-1$ after the Wnt-regulated EMS division (Bei, Mello et al 2002). In the EMS division, SYS-1 is enriched in the posterior E nucleus, but not that of the anterior MS (Fig. 5A-B). Analyzing nuclear enrichment of GFP-tagged SYS-1, we were able to observe increased nuclear GFP

signal in both the E and MS daughters in analyzed trafficking mutants (Fig. 5C). Interestingly, daughters affected by both *ecps-1(RNAi)* and *dlc-1(RNAi)* exhibit significant enhancement of nuclear enrichment in each of the two daughter cells (Fig 5C), suggesting that these treatments act negatively on SYS-1, despite opposing effect on SYS-1 centrosomal enrichment. Following loss of negative centrosomal regulation, these data suggest that daughter cell SYS-1 inheritance is increasing and thus affecting the bias of the EMS asymmetric division, increasing the likelihood of unsignaled daughter cells adopting the signaled endoderm cell fate.

Having established an increase in nuclear GFP::SYS-1 in dynein subunit depletion, even in cells not signaled to maintain activating SYS-1, we turned to the effect of SYS-1 expression on target gene activation. Specifically, we used the endogenous promoter of W β A target gene *end-1* to drive GFP-tagged Histone 2B in order to evaluate the activity of SYS-1 immediate downstream targets as the signaled E cell established the endoderm tissue expansion from 2 to 4 to 8 cells (E+1, 2, or 3 divisions, respectively). Normally, this strain exhibits additional expression of the *end-1* promoter in <10% of embryos, a frequency which decreases further when SYS-1 is depleted via RNAi (Fig. 5D). In embryos depleted of either dynein subunits or RSA-2 by RNAi, however, we observed a different pattern. In these embryos, misregulation via dynein depletion appeared to increasingly overwhelm SYS-1 negative regulation in successive divisions, such that each generation after SYS-1 dependent specification increasingly expresses the *end-1* promoter driven endodermal reporter outside of the EMS lineage (Fig. 5D). For instance, in *dhc-1(RNAi)* we observe only 3% of embryos mis-expressing one cell division after the specification of the E cell type (E+1), which increases to 25% by the next division (E+2), and over 83% three divisions after specification (E+3).

We also investigated larval ACDs to determine the effect that extra transcriptionally active SYS-1 has on organismal development. We examined the seam cells (SCs) and distal tip cells (DTCs) because of

their sensitivity to SYS-1 dependent gene activation (Baldwin, Phillips et al 2016; Baldwin and Phillips 2018; Lam and Phillips 2017; Mila, Putzke et al 2015). In wild-type animals, DTC lineages undergo asymmetric cell division during L1 to give rise to two DTCs, while *sys-1* mutants and animals overexpressing SYS-1 show loss or gain of DTCs, respectively, due to symmetric cell division (Fig 6A) (Miskowski, Kimble et al 2001; Siegfried, Kimble et al 2004; Kidd, Kimble et al 2005; Chesney, Kimble et al 2009). To further sensitize these cells for changes in SYS-1 dependent cell fate changes, we examined strains containing additional, transgenic SYS-1 driven by the heat shock promoter. In animals that are not subjected to heat shock, dynein depletion only rarely induced development of more than the endogenous 2 distal tip or 16 seam cells (Fig. 6B, 7B). In heat shocked animals, excess SYS-1 protein can overwhelm canonical SYS-1 regulation to develop as many as 6 DTCs (Fig. 6D). While heat shock-induced overexpression is sufficient to cause a notable shift towards the signaled cell fate, dynein subunit depletion was able to significantly enhance the effect of heat shock (Fig. 6C). Depletion of DHC-1 or ECPS-1 at the time of the somatic gonadal precursor division was sufficient to induce rare but wild-type-unobserved additional DTCs (0.3% of animals for these 2 treatments, N= 394, 363, respectively). Adding heat shock-overexpressed SYS-1 enhanced the penetrance of ectopic DTCs, with DHC-1 and ECPS-1 depletion resulting in populations where 11.9% and 6.8% of animals, respectively, develop more than 2 DTCs. Depletion of RSA-2, uncoupling SYS-1 from centrosomes, and depletion of a dynein light chain, DYLT-1, further enhanced these misspecifications, increasing the proportion of the population with ectopic DTCs from 26.6% in wild type to 33.6% or 51% after RNAi depletion, respectively (Fig. 6D).

The SC lineage also undergoes several W β A-dependent ACDs throughout larval development (Fig 7A). Similarly, in the SC tissue, depletion of dynein light chains alone is sufficient to increase the frequency of ectopic SCs from 3.3% of larvae with ectopic SCs in control RNAi conditions to 11.7% or 10% of animals as seen in DYLT-1 and DLC-1 depletions, respectively (Fig. 7B). These misspecifications are further exacerbated by SYS-1 overexpression, wherein more than 60% of the *dylt-1(RNAi)* larvae

have greater than wild type seam cell numbers, developing as many as 21 SCs, (Fig. 7C). These observations collectively support SYS-1 centrosomal enrichment by dynein as a mechanism to reinforce SYS-1 function in specifying SYS-1-dependent cell fate decisions. Therefore, compromising the centrosomal SYS-1 regulatory system by dynein depletion is rarely sufficient to induce misspecifications in otherwise wildtype animals, but notably enhances the effects of ectopic SYS-1 accumulation. Therefore, dynein-dependent centrosomal regulation of transcriptional coactivator SYS-1 increases the robustness of W β A-regulated cell fate decisions after ACD.

Discussion

Precise control of cell fate determinants is critical for normal development and homeostasis, particularly in the rapidly dividing cells of the early embryo. Regulation of these factors includes the distribution and inheritance of these determinants by daughter cells. Here we demonstrate that centrosomal negative regulation of *C. elegans* β -catenin SYS-1 is enhanced during asymmetric division by trafficking to centrosomes by the microtubule motor dynein. Depletion of individual subunits of the dynein motor and associated proteins is sufficient to reduce the levels of centrosomal SYS-1, and temporally specific ablation of dynein function by *dhc-1(ts)* was sufficient to remove observable SYS-1 nearly completely and disproportionately from centrosomes. However, some methods of disrupting dynein/microtubule trafficking result in an unexpected increase in centrosomal accumulation of SYS-1 protein. These increases, caused by microtubule disruption via Nocodazole and depletion of SYS-1 negative regulator adapter ECPS-1 phenocopy centrosomal enrichment seen in disruption of proteasome activity, and maintain a SYS-1 immobile fraction like that seen in *rpt-4(RNAi)* depletion. We therefore propose a model wherein both SYS-1 and regulatory components of the ubiquitin-proteasome system, are dependent on microtubule-mediated trafficking to centrosomes by the dynein complex in order to appropriately reinforce SYS-1 asymmetric regulation across daughter cells (Fig. 8).

In this model, SYS-1 enriches at centrosomes and is degraded or marked for degradation before leaving the centrosome. Rather than a static centrosomal enrichment, this model entails continual trafficking by microtubule motors to maintain an observable centrosomal pool of SYS-1. The observable CEI therefore measures a steady state of centrosomal enrichment at centrosomes, wherein SYS-1 is localized to centrosomes more rapidly than it is marked and cleared. SYS-1 therefore becomes visibly enriched at centrosomes compared to cytoplasmic levels. This model predicts that even subtle disruptions to microtubule mediated trafficking could limit the ability of cells to maintain wild type SYS-1 centrosomal enrichment, assuming the SYS-1 clearance rate remains unchanged.

Interestingly, the *rsa-2(RNAi)* enhancement of kinetochore localization of SYS-1 in this model is consistent with ‘traffic jams’ observed in microtubule mediated trafficking (Leduc, Howard et al 2012). In these cases, crowding motors by increasing the local concentration of motor proteins, introducing microtubule-binding obstacles, or, importantly for our model, preventing motor dissociation limits the activity of motor proteins (Nam and Epureanu, 2017; Ferro, Yildiz et al 2019). Evidence of traffic jams in vivo have been shown in neuronal microtubule transport, as neuronal kinesin and its cargo synaptotagmin accumulate when trafficking is disrupted by hypomorphic kinesin alleles. The disrupted motor complexes then accumulate upstream of the trafficking impediment, in an accumulation of both motor and cargo (Hurd and Saxton 1996).

The kinetochore localization of SYS-1 is consistent with a traffic jam model, thus making SYS-1 trafficking by microtubule motors a more attractive model. Decreasing centrosomal SYS-1 localization by *rsa-2(RNAi)* increases SYS-1 protein in resulting daughter cells (Vora and Phillips 2015). A severely limited centrosomal recruitment of SYS-1 in *rsa-2(RNAi)* may also suggest problematic SYS-1/motor protein unloading upon reaching the MTOC centrosome. Similarly, $P_{pie-1}::GFP::SYS-1$, which appears overexpressed compared to $P_{sys-1}::VENUS::SYS-1$, may exacerbate SYS-1 traffic jams by increasing the cytoplasmic SYS-1 pool. Indeed, the combination of $P_{pie-1}::GFP::SYS-1$ and *rsa-2(RNAi)* results in a further

increase in the incidence of SYS-1 localization to kinetochore microtubules, suggesting increased SYS-1 levels coupled with decreased centrosomal capturing may reveal an otherwise transient pool of SYS-1 en route to the MTOC centrosome. Inefficient SYS-1 capture in *rsa-2(RNAi)* embryos likely further crowds SYS-1-containing microtubule motor complexes into ‘traffic jams’ because, in embryos lacking a mechanism to anchor SYS-1 to the centrosome, SYS-1 is trafficked toward centrosomes more quickly than it is captured there and processed. This shift results in an observable SYS-1 accumulation at regions of high microtubule occupancy, such as kinetochore microtubules and the cell cortex, both of which are increased after RSA-2 depletion.

Conversely, centrosomal protein processing by either post-translational modifications or degradation via the proteasome likely requires the trafficking of fewer, yet reusable enzymes. The disparity between substrate vs enzyme trafficking requirements would suggest that clearance of centrosomal substrates is less vulnerable to mild or short-term disruptions to trafficking than the maintenance of a centrosomal substrate pool. Upon long-term or severe disruptions to microtubule trafficking, a trafficking requirement for clearance mechanisms becomes apparent. Our model predicts that disruption of either SYS-1 localization (inefficient trafficking to the site of regulation) or disrupting the localization of a member of the Ubiquitin-Proteasome System (UPS) (where SYS-1 is trafficked and accumulates, but centrosomal regulation/degradation is ineffective) both result in increased SYS-1 dependent gene activation and differentiation in daughter cells, consistent with excessive inheritance. While this system of centrosomal localization and clearance of SYS-1 does not appear necessary for most W β A differentiation events, disruptions to SYS-1 trafficking or processing limit the system’s ability to tolerate perturbations. That is, SYS-1 centrosomal localization generates robustness in SYS-1 dependent cell fate decisions (Fig. -7) due to parallel trafficking mechanisms; disrupting either trafficking mechanism results in excessive SYS-1 dependent activity in daughter cells of mothers with either increased or decreased SYS-1 CEI.

Why does no single dynein disruption treatment fully recapitulate the *rsa-2(RNAi)* phenotype?

While we see individuals within several dynein knockdown treatments with nearly *rsa-2(RNAi)*-like SYS-1 centrosomal uncoupling, these populations also include embryos with roughly wild type centrosomal SYS-1 enrichment. However, given the severely deleterious nature of dynein subunit loss-of-functions (Sönnichsen, Neumann et al 2005) and the variability of feeding RNAi knockdowns as compared to null mutant strains (Tavernarakis, Driscoll et al 2000), it seems likely that our significant decreases in SYS-1 centrosomal recruitment represent a phenotypically incomplete knockdown. Surviving embryos then only exhibit hypomorphic dynein function, insufficient to induce catastrophic defects in spindle assembly but sufficient to affect SYS-1 centrosomal enrichment. However, it is also possible that there is partial redundancy between the SYS-1 relevant functions of the dynein motor. Introducing *dhc-1(ts)* overcomes either incomplete knockdown or redundancy since a thorough dynein disruption is able to robustly uncouple centrosomal SYS-1.

Why, then, do we observe an increase in centrosomal SYS-1 steady state upon either long term lower dose nocodazole-mediated knockdowns of trafficking or by RNAi depletion of trafficking subunit ECPS-1? In response to these data (Fig. 3), we propose the additional trafficking of a SYS-1 negative regulatory component to this model. Disrupting the trafficking of centrosomal SYS-1 negative regulators should result in a previously unseen increase in centrosomal SYS-1 steady state while maintaining a similar effect on downstream gene expression and cell fate decisions, as seen above (Fig. 3-7). However, the difference in SYS-1 enrichment between most dynein depletions and that of ECPS-1 or nocodazole treatment suggests a differential efficiency in trafficking or centrosomal capture rate between SYS-1 and its negative regulators, such as the proteasome. Most disruptions to the dynein motor that reduce SYS-1 centrosomal accumulation, mediated by maternally fed RNAi, are consistent but likely incomplete because of their requirement for oogenesis and early embryonic viability. Relatively mild dynein knockdowns resulting in viable embryos, therefore, are likely able to form dynein complexes, but at a far

reduced rate. These dynein-limited embryos should therefore decrease the rate of continual SYS-1 trafficking while not preventing the centrosomal localization of an occasional regulatory components, perhaps of the Ubiquitin-Proteasome System (UPS). The reusable tagging or proteolytic machinery is likely sufficient to enzymatically process SYS-1 that still localizes to the centrosome, but the rate of incoming SYS-1 is significantly reduced. Similarly, depletion of dynein function by *dhc-1(ts)* effectively depletes all dynein function but can only do so for brief portions of mitosis without inducing mitotic spindle collapse.

While dynein depletions limit the volume of trafficking by limiting the number of properly constructed dynein motors, they do not limit the ability of an individual, functional dynein motor to traffic. The experimental conditions that increase the steady state of centrosomal SYS-1, Nocodazole and *ecps-1(RNAi)*, instead limit the scope of dynein cargo trafficking. ECPS-1, putative adapter between dynein and the proteasome, increases the steady state of both SYS-1 and additional centrosomal proteasome target ZYG-1. Increased enrichment of both proteins is consistent with the conserved role of ECM29 for ECPS-1, and results in embryos that are not inhibited for overall dynein function but limited in their ability to traffic proteasome. Nocodazole treatment similarly does not directly affect the processivity of dynein complexes that form on the comparatively limited microtubule network. However, it does severely affect the reach of the microtubule network itself (Fig. 3A). Our data therefore suggests that the proteasome requires less continual reinforcement by microtubule-mediated trafficking but does require more extensive microtubule formation than is allowed in our Nocodazole-treated embryos.

A role for trafficking in both SYS-1 and its regulatory components, then, suggests a differentially maintained steady state interaction for the proteasome and centrosomal SYS-1. While SYS-1 is rapidly and continuously trafficked, it is similarly rapidly processed by centrosomal proteasome. That is, the apparently rapid processing of centrosomal SYS-1 also requires rapid dynein-mediated flux to maintain

its centrosomal steady state. Any disruption to dynein processivity will therefore allow slower SYS-1 centrosomal enrichment to be overcome by its centrosomal processing, reducing the SYS-1 CEI by slowing the transfer of SYS-1 from cytoplasm to centrosome for degradation. By comparison, the proteasome or ubiquitin ligases endure at the centrosome, continually processing centrosomal targets. Our proposed model therefore predicts that short term or partial disruptions to the dynein motor (Fig. 8, red inset) allow for the eventual establishment of a centrosomal proteolysis pathway, which may affect both SYS-1 and proteasome localization, but nevertheless result in decreasing steady state levels of centrosomal SYS-1 (and presumably other proteasome targets that require active trafficking) as occasional proteasome centrosomal localization overcomes the impaired localization of SYS-1. Conversely, long term systemic disruptions to microtubule-mediated motors like that seen in Nocodazole treatment may still capture SYS-1 in their comparatively limited microtubule network, while being disproportionately deprived of the larger proteasome complex (Fig. 8, blue inset).

Our model that both SYS-1 and its regulators are trafficked was supported but the identification of a trafficking component that affects one but not both arms of our trafficking model. Most of our perturbations, generally affecting components of the dynein machinery, may exert their effects by either detaching SYS-1 from the motor or by sabotaging all function of the motor complex. However, the specificity of our two transport mechanisms was exhibited by one dynein-protein interaction, via depletion of ECPS-1/the putative ECM29 subunit of the motor. Unlike the other subunits presenting strong SYS-1 phenotypes, ECM29 has not yet been implicated in dynein function beyond its adaptor role but has notable effects on proteasome localization and response to stress (Gorbea, Rechsteiner et al 2010; Haratake, Chiba et al 2016). Because *ecps-1(RNAi)* phenocopies the centrosomal GFP::SYS-1 accumulation seen in proteasome knockdown via *rpt-4(RNAi)* as well as microtubule network disruption via Nocodazole treatment, we propose that these observations support a role for proteasomal localization as well as function for proper centrosomal clearance of SYS-1 (Fig. 8).

While this model and our experiments to test it have been largely centered on SYS-1/ β -catenin, many developmental and temporally regulated proteins are known to pass through the centrosome (Vora and Phillips 2016; Brown, Welch et al 1994; Johnston, Kopito et al 1998; Huang and Raff 1999; Reck-Peterson, Carter et al 2018), as the centrosomal proteasome is far from SYS-1-specific. It is not unreasonable, then, to presume that other proteins may benefit from dynein trafficking to something as short-lived and yet influential to daughter cell inheritance as the mitotic centrosome. Particularly early in eukaryotic development, subtle changes in the inheritance of cell fate determinants like SYS-1 can have a profound effect on the ability of an organism to respond to environmental insults. For example, the asymmetric nuclear localization of SYS-1 indicative of differential cell fate has been shown to arise early, less than 70 seconds after the establishing division (Baldwin and Phillips 2014; Vora and Phillips 2015). We therefore propose that an active dynein mediated trafficking, at least for cell fate determinants like SYS-1, could provide a universal buffering against excessive inheritance and consequentially rare but deleterious cell fate changes.

Materials and Methods

Strains

Strains were maintained on Op50-inoculated NGM plates at 15° (BTP51, BTP220) or 25° C (TX964, BTP51, LP563, TX691) using typical *C. elegans* methods. These strains contained the following transgenes and alleles: **N2** (wild type); **TX964** (unc-119(ed3) III; him-3(e1147) IV; teis98 [Pie-1::GFP::SYS-1]); **BTP51** (unc-119(ed3) III; tjl571 [pie-1 promoter::mCherry::H2B, pie-1 promoter::2x mCherry::tbg-1, unc-119(+); qIs95 III] ; **BTP220** (unc-119(ed3) III; tjl571 [pie-1 promoter::mCherry::H2B, pie-1 promoter::2x mCherry::tbg-1, unc-119(+); qIs95 III; dhc-1(or195) I.] ; **TX691** (unc-119(ed3) III; tels46 [pRL1417; end-1p::GFP::H2B + unc-119(+)] ; **OC341** (unc-119(ed3) III; bsIs8[pMS5.1:unc-119(+)] pie-1- gfp-zyg-1C-terminus]); **BTP93** (*wIs78[SCMp::GFP + ajm-1p::GFP + F58E10 (cosmid) + unc-119(+)]*

IV; uiwEx22[pHS::sys-1]; BTP97 (qls56[lag-2p::GFP + unc-119(+)] V; uiwIs3[pHS::sys-1]; AZ235 (unc-119(ed3) III; ruls48[Ppie-1::gfp::tbb-1]; TH107 (P_{pie-1}::RSA-2:: GFP)

RNAi

To perform RNAi knockdown of target genes, we used HT115 bacteria containing the pL4440 plasmid with a T7-flanked target gene insert. Most of these were obtained from a library supplied by Ahringer (Kamath and Ahringer 2003). Those which were otherwise obtained (*dlc-1*) used target gene-flanking primers with *C. elegans* cDNA to generate a new insert. These bacteria were used to seed IPTG-containing, inducing plates as described previously. Worms were generally plated to RNAi after Sodium Hypochlorite Synchronization as L1's. More deleterious knockdowns (*dlc-1*, *dhc-1*) were washed from OP50 plates at 24 hours before imaging (L3/L4) or 12 hours before imaging (late L4/early adult) (Timmons 2006; Bekas and Phillips 2020). DLC-1 was derived using RT primers and digested with Xho I and HIND III SEQUENCING 7/31

Compound Microscopy, CEI, and Image Processing

To obtain samples, mothers were anesthetized with 250 μ M levamisole and dissected *via* a clean surgical blade. Embryos were then transferred to a 2% agarose pad and imaged on a Zeiss Axio Imager.D2. All exposure times were kept constant. Centrosomal measurements were collected from the mitotic P1 cell of the early embryo during anaphase, at approximately similar inter-polar distance (Fig. S5). Images were exported as uncompressed TIF's, and fluorescence intensity was determined by measuring the mean fluorescence of centrosomal puncta in ImageJ. The mean intensity of the remaining embryonic cytoplasm was subtracted from the centrosomal mean to produce the CEI measurement, and identical measurements on 2x mCherry::tbg-1 were used for TEI. END-1 family nuclei were assayed via the TX691 strain. GFP-tagged Histone intensity was measured relative to the cytoplasmic autofluorescence.

Temperature Sensitive Allele Imaging

BTP51 and BTP220 were maintained together at 15°C and were imaged as above. Ambient temperature in the microscope room was decreased to ~18°C for ‘permissive’ imaging and upshifted to ‘restrictive’ 26°C by moving slides to a Pecon Tempcontroller 2000-2 heated stage. Embryos were imaged at the two temperatures sequentially, with approx. 45 seconds between upshift and image, or were imaged as populations at either the permissive or restrictive temperature.

Nocodazole Treatment

In order to treat embryos before formation of the drug-impermeable eggshell, we exposed late L4’s to a low dose (approx. 83 μ M, 25 μ g/mL) nocodazole dissolved in 2% DMSO for 16 hours. Embryos were then concentrated by one minute centrifugation at 1000xG and imaged as described above. Permeabilization of embryos was performed as described in Carvalho, Oegema et al 2011. Imaging was done as described above, but included 4% sucrose and 0.1M NaCl to the 2% agar pads to bolster embryo stability (Greenan, Hyman et al 2010; Gönczy, Schnabel et al 1999).

Confocal Microscopy and Fluorescence Recovery After Photobleach

Confocal microscopy was performed via a Leica SP8 HyD detector system. The objective used was a 63x HC PL APO CS2 objective with N.A. = 1.4, utilizing type F immersion oil. Each image analyzed consisted of the sum of 35 z-images across the embryo, each slice approximately 0.35 μ m thick. FRAP was performed on the same system. Each embryo was imaged twice before bleaching, beginning as soon as possible after NEBD. The ROI, an approximately 5 μ m square, was then bleached at an intermediate focal plane with 100% laser power for 100 iterations. Images were taken as above, once every 7 seconds. Fluorescence intensity was measured on summed slice Z-projections in FIJI via the mean fluorescence intensity of ROIs drawn around relevant locales – either centrosome or the entire

dividing cell. Recovery was assayed by subtracting the ROI post-bleach intensity from each measurement and evaluating it as a percentage of the pre-bleached centrosomal intensity.

Heat Shock-Induced SYS-1 Overexpression

BTP93 and BTP97 worms were exposed to brief 33°C heat shocks at relevant developmental timepoints to drive SYS-1 overexpression concurrently with signaled cell fate specification. For DTCs, BTP97 was subjected to 1-hour heat shock, 30-minutes recovery, and 30-minutes additional heat shock beginning 11.5 hours after plating. For SCs, BTP93 was subjected to 2-hour heat shock, 30-minute recovery, and additional 1-hour heat shock beginning 26 hours after plating. Both tissue types were assayed 50 hours after plating.

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FIGURE LEGENDS

Figure 1:

Rationale and methodology for microtubule motor RNAi Screen. A) GFP::SYS-1 can be occasionally observed at regions corresponding to DHC-1 mitotic accumulation. This is particularly enhanced in centrosomally uncoupled *rsa-2(RNAi)* embryos. The frequency of GFP::SYS-1 enrichment at these locales in *lacZ(RNAi)* and *rsa-2(RNAi)* treatment is distinguished by color. A') GFP::SYS-1 at kinetochore microtubule (white arrowhead) and cortex(black arrowhead) of the dividing *C. elegans* early embryo. B) Demonstration of CEI and TEI calculation. CEI was calculated in FIJI by subtracting the mean pixel intensity of a cytoplasmic ROI encompassing the proximal half of the dividing cell, approximated by the light blue square, from a circular centrosome ROI, approximated by the white square. B') Expanded view of ROI's as defined in B. B'') Graphical representation of the raw centrosomal and CEI adjusted fluorescence intensity for the control (Left) and Affected (right) embryos in B. Control (*lacZ(RNAi)* treated) exhibits both a high raw centrosomal GFP::SYS-1 and CEI. Affected (*rsa-2(RNAi)* treated) exhibits an intermediate raw GFP::SYS-1, but severely decreased CEI, better reflecting its visual failure to increase centrosomal GFP::SYS-1 notably above the bulk of cytoplasmic representation. C) Example VENUS::SYS-1 CEI (C) and CEI/TEI (C') of controls and indicated dynein light chain depletions. Width of the violin plot corresponds to a histogram of observation density. The median is indicated by the central white dot, with the interquartile range indicated by the thick black line, and 1.5x additional Interquartile range indicated by the thin black line. N= *lacZ(RNAi)*, 72; *dlc-1(RNAi)*, 74; *dylt-1(RNAi)*, 52; *rsa-2(RNAi)*, 22 * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$ by Chi-square test (A) or Student's t-test (C-C').

Figure 2:

The dynein heavy chain Temperature Sensitive (*dhc-1(ts)*) Allele mediated loss of dynein function has a disproportionate effect on SYS-1 localization. A) Diagram of the Dynein motor, and hypothetical model of its role in SYS-1 trafficking. Relative location of *dhc-1(ts)* lesion indicated. B) Representative centrosome images of VENUS::SYS-1 and g-Tubulin::mCherry in wildtype and *ts* allele-containing embryos C) embryos at comparable cell cycle phase before (top) and after (bottom) temperature upshift. D) SYS-1 CEI and D') SYS-1 CEI per TEI in embryos containing *dhc-1(ts)* , to control for differences in centrosome assembly, standard error depicted. N= 44, 31, 38, 25 in D and D' columns, left to right. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$ by Student's t-test.

Figure 3:

Nocodazole mediated disruption of microtubules enhances centrosomal GFP::SYS-1 enrichment A) Representative images of GFP::TBB-1 in DMSO and Nocodazole treated embryos. White arrowheads indicate astral microtubules (left) and the corresponding lack or dysregulated region in nocodazole treatment (right) B) Quantification of, and C) Representative images of similarly treated GFP::SYS-1 embryos. For B, N=36 DMSO and 46 nocodazole treated embryos. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$ by Student's t-test. D) CEI/TEI as evaluated with combinatorial Wildtype (WT) or *dhc-1(ts)* allele for Dynein Heavy Chain, and treatment with 2% DMSO vehicle media or 2% DMSO + nocodazole media. Measurements at the permissive and restrictive temperature are differentiated by blue and red coloring, respectively. Error bars are SEM. E) Representative P_{SYS-1} VENUS::SYS-1 and mCh::g-Tubulin for each treatment in D, respectively.

Figure 4:

Microtubule-mediated processes can also stabilize centrosomal SYS-1. A) Treatment with *ecps-1(RNAi)* enhances centrosomal SYS-1 accumulation, N=48 *lacZ(RNAi)*, N=12 *rsa-2(RNAi)*, N= 124 *ecps-1(RNAi)*. B)

lacZ(RNAi) and *ecps-1(RNAi)* representative centrosomes in grayscale and intensity dependent LUT. C) Putative dynein and proteasome adaptor ECPS-1 also appears to also enhance centrosomal ZYG-1 accumulation, by GFP::ZYG-1c CEI (N=31 untreated, N=19 *ecps-1(RNAi)*, N= 20 *rpt-4(RNAi)*, 14 *rsa-2(RNAi)*) and D) Representative GFP::ZYG-1c centrosomal images in grayscale and intensity dependent LUT. E) Quantified recovery of GFP::SYS-1. Values indicated are the ratio of bleached centrosomes to their pre-bleach value, normalized such that the post-bleach value, t = 0, is 0 GFP::SYS-1 intensity. Error bars are SEM. (F-F'') representative FRAP montage of 2nd division P1 cells in indicated embryos. Two pre-bleach, immediately consecutive timepoints are followed by the 1st post-bleach image at time '0' and images every 7 seconds thereafter indicated top left. * - p<0.05, ** - p<0.01, *** - p<0.001 by Student's t-test. One-sided Student's T, asterisk indicates p-value from *lacZ(RNAi)* in E-F.

Figure 5:

Effect of Dynein Depletion on nuclear SYS-1 Localization and Gene Expression in EMS Descendant

Cells. A) The EMS precursor (embryo ventral cell) divides, receiving Wnt and Src specification and orientation cues, respectively (red arrow). These daughters give rise to posterior, signaled E and anterior, unsignaled MS daughter cells. Wildtype specification results in depicted approximate GFP::SYS-1 localization pattern specifically enriched in the signaled daughter nucleus. B) Representative images of wildtype and *ecps-1(RNAi)* treated embryos. Orange box encloses the unsignaled MS nucleus, Blue box encloses the signaled E nucleus. C) Quantification of GFP::SYS-1 nuclear enrichment in indicated dynein RNAi knockdowns. C') Mean difference in nuclear GFP::SYS-1 enrichment from quantification in C. Error bars are SEM. D) Embryos ectopically expressing pEND-1::GFP::H2B in cells not descended from the E lineage in the first (orange) second (blue) and third (green) lineage after the founding of the lineage (the 2, 4, and 8 cell E descendants, respectively). D') Representative embryos exhibiting WT (all *lacZ(RNAi)*-

treated, left) and ectopic (*ecps-1(RNAi)*- and *2xdhc-1(RNAi)*- treated for 1, 2, and 3 generations after E founding, respectively, right) pEND-1::GFP::H2B

Figure 6:

SYS-1 dysregulation via dynein knockdown can cause rare distal tip cell fate conversions. A) Diagram of *C. elegans* distal tip cell signaled lineage specification in the somatic gonadal precursor. B) Representative image of WT (top) and ectopically induced (bottom) distal tip cells (DTCs). To distinguish DTC's from pLAG-2 driven vulval expression, DTCs are marked with white arrowheads. C) Number and relative frequency of DTCs via anatomical marker in untreated animals and in backgrounds with RNAi depletion of various dynein subunits. D) Number and relative frequency of DTCs marked by anatomical marker when SYS-1 is overexpressed via a heat shock-driven transgene in both untreated animals and in backgrounds with RNAi depletion of various dynein subunits. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ by one-sided Mann-Whitney test

Figure 7:

SYS-1 dysregulation via dynein knockdown can cause rare seam cell fate conversions. A) Diagram of *C. elegans* seam cell (SC) signaled lineage. B) Representative image of WT (top) and ectopically induced (bottom) SCs. Likely ectopic SC exhibit spatially distinct 'doublets', highlighted here in brackets. C) Number and relative frequency of SCs via anatomical marker when in untreated adults and in backgrounds with RNAi depletion of various dynein subunits. D) Number and relative frequency of SCs via anatomical marker when SYS-1 is overexpressed via a heat shock-driven transgene in both untreated larvae and in backgrounds with RNAi depletion of various dynein subunits. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ by Mann-Whitney test

Figure 8:

Proposed model. Speculation into the various processes by which SYS-1 and its regulators appear to be localized to mitotic centrosomes. Both SYS-1 and some component of its centrosomal proteasome-dependent negative regulators, here represented by purple circle, demonstrate some dependence on microtubule mediated trafficking. While various dynein subunits are requisite components of a functional dynein complex, the negative effect of their depletion on SYS-1 centrosomal steady state indicates a preferential or more frequent trafficking of SYS-1. Relatively rare proteasome trafficking events mediated by ECPS-1 meanwhile, are sufficient to maintain appropriate SYS-1 centrosomal enrichment. This less efficient proteasomal trafficking, however, renders it vulnerable to Nocodazole-mediated decrease in overall microtubule trafficking/network formation efficiency. Red inset depicts decreased processivity or cargo binding capacity induced by dynein light chain RNAi depletions. Blue inset depicts depletion of adapter ECPS-1 and resulting depletion of proteasome-trafficking specific dynein complexes, while not otherwise affecting the microtubule motor.

A **B** **C** **D** **D'**

B

# of SCs	15	16	17	18	19	20	21	
<i>P_{hs}-sys-1</i> (no <i>hs</i>); <i>lacZ(RNAi)</i>	1.7%	95%	3.3%					N=60
<i>P_{hs}-sys-1</i> (no <i>hs</i>); <i>dlc-1(RNAi)</i> *		90%	8.3%			1.7%		N= 60
<i>P_{hs}-sys-1</i> (no <i>hs</i>); <i>dylt-1(RNAi)</i> *		88.3%	10%			1.7%		N= 60

C

# of SCs	15	16	17	18	19	20	21	
<i>P_{hs}-sys-1</i> (w/ <i>hs</i>); <i>lacZ(RNAi)</i>	1.7%	80%	13.3%	5%				N=60
<i>P_{hs}-sys-1</i> (w/ <i>hs</i>); <i>dlc-1(RNAi)</i> **	1.7%	58.3%	33.3%	5%	1.7%			N= 60
<i>P_{hs}-sys-1</i> (w/ <i>hs</i>); <i>dylt-1(RNAi)</i> ***	1.7%	38.2%	26.7%	11.7%	11.7%	5%	5%	N= 60