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Jina Lee April 12, 2022

Exploring the Regulation of *cen* mRNA Localization to Centrosomes

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Science with Honors

Department of Biology

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Abstract

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The centrosome plays a crucial role in maintaining genomic stability. Aberrant centrosome activities can manifest in mitotic errors, developmental disorders, and are associated with poor cancer prognosis. The activities and functions of centrosomes are regulated by the pericentriolar material (PCM), and previous studies have identified many different mRNAs localizing to the PCM. Understanding the role and function of mRNA at the centrosome is a topic of active investigation. Work from our laboratory recently demonstrated the localization of *centrocortin* (*cen)* mRNA is required for mitotic fidelity. The disruption of *cen* mRNA localization causes errors in cell division. However, the specific mechanism underlying *cen* mRNA localization to and regulation of the centrosome is not clear. Here, we investigated which *cis* and *trans* factors contribute to the localization of *cen* mRNA to the centrosome. We hypothesize that the RNA-binding protein Egalitarian (Egl) links *cen* mRNA to the motor protein dynein to transport *cen* to the centrosomes. To test this hypothesis, we examined the *trans*- and *cis*-acting elements required for *cen* mRNA localization using *cen* truncations and *egl* mutant lines. Identification of the mechanisms underlying *cen* mRNA localization to the centrosome will ultimately allow us to deepen our understanding of mRNA localization and centrosome regulation, aspects of which may be deregulated in the disease setting.

Exploring the Regulation of *cen* mRNA Localization to Centrosomes

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Acknowledgements

I would like to thank the members of the Lerit lab for providing me with a pathway and help to perform this work. The completion of this thesis would not have been possible without the support I have received from the Lerit lab. I appreciate the Gonsalvez lab for providing the *egl* RBD mutant line. Additionally, I would like to express gratitude to my research mentor, Dr. Hala Zein-Sabatto, and my research advisor, Dr. Dorothy Lerit, for exceptional supervision during this research.

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Introduction

The localization of mRNA plays a central role as a mechanism to control cellular function. Thus, it is deeply conserved across many organisms. Aside from functioning as a template for protein expression, mRNA allows cells to define subcellular compartments, form specialized structures, and accomplish precise functions. Proper mRNA localization is crucial in ensuring proper coordination of cellular functions. It not only reduces the energy cost of transporting proteins by repeatedly using mRNA molecules as an energetically favorable template for protein expression, but it also prevents proteins from reaching a detrimental compartment (Zein-Sabatto and Lerit, 2021). Therefore, understanding the mechanism and players involved in mRNA localization is crucial to our understanding of cellular structure and function.

mRNA localizes to defined cellular compartments and organelles, such as the centrosome. The centrosome is required for error-free mitosis, and it comprises a central pair of centrioles embedded in a mass of proteins known as the pericentriolar material, or PCM. The PCM is an organized, yet dynamic, ring-like matrix of proteins (Mennella et al., 2014). This dynamic collection serves as a platform for protein complexes that regulate the centrosome. These proteins serve as cell cycle regulators, signaling molecules, and organize the nucleation of microtubules. Thus, the microtubule-nucleating activities of the centrosome are regulated by the PCM, and deregulated PCM contributes to centrosome aberrations (Conduit et al, 2015).

However, the nature of PCM is remains an outstanding question. Classical experiments show the centrosomes form the poles of the bipolar mitotic spindle during mitosis, and those spindle poles ensure the division and formation of new daughter cell (Rappaport, 1961). Through

high content mRNA in situ hybridization screening efforts, we now know that a subset of mRNAs localizes to spindle poles, and their distribution correlates with the cell cycle, suggesting the localization of mRNA to centrosomes is a dynamic process (Lecuyer et al., 2007; Ryder et al., 2020; Wilkie and Davis; 2001). Yet, the function and mechanisms underlying the localization of mRNA to centrosomes are still under investigation. Understanding how mRNAs localize to centrosomes and their biological functions will likely augment our understanding of centrosome regulation, aspects of which may be deregulated in cancer cells or other disease states.

General principles guiding mRNA localization were identified by classical studies in oocytes, early embryos, and polarized neurons (Mili and Macara, 2009). Intrinsic components required for mRNA localization are typically characterized by *cis*- and *trans*-acting elements, where *cis*-acting elements are sequences and/or structures with the mRNA that function as a "zip code" that determines where the mRNA will localize (Jambhekar and Derisi, 2007; Meer et al., 2012). These *cis*-acting elements serve as binding sites for *trans*-acting proteins. *Trans*-acting regulatory elements include RNA-binding proteins and motor proteins recognize *cis*-acting elements within the RNA transcript (Gerstberger et al., 2014). These components allow mRNAs to localize their sites through active transport (Bergsten and Gavis, 1999; Bullock, 2011).

The microtubules nucleated by the centrosome are polarized with minus ends embedded within the PCM and plus ends facing out into the cytoplasm. The polarized orientation of microtubules facilitates various cell movements, including intracellular transport. Kinesin and dynein are microtubule motor proteins that move along the polarized microtubules (Cooper, 2000). The polarized structure of microtubules is derived from the tubulin subunits, which comprise heterodimers of alpha- and beta-tubulin (Alberts, 2008). Cytoplasmic dynein- and dynactin- mediated transport towards the minus ends of microtubules (Wilkie and Davis, 2001).

Therefore, we posit that this intracellular network of dynein-mediated active transport contributes to centrosomal enrichments of mRNA. Egalitarian (Egl) is an RNA-binding protein for the dynein motor, and it helps attach RNA cargoes to the dynein motor protein (Dienstbier et al., 2009). Previous work also shows Egl is required for early *Drosophila* oocyte microtubule organization (Huynh and Johnston, 2000). Egl, and its binding partner Bicaudal (BicD), associate with dynein light chain and dynein complexes, and these adaptor proteins and molecular motors interact with each other to localize minus-end directed cargoes, including mRNAs (Dienstbier et al., 2009; Goldman et al., 2019; Liu et al., 2013; Schlager et al., 2014).

Among the mRNAs localizing to the centrosome, *centrocortin* (*cen)* mRNA is the only one whose function was directly examined. The localization of *cen* mRNA was mistargeted to the *Drosophila* syncytial embryo anterior pole by fusing the *cen* coding sequence to the *bicoid* (*bcd*) 3'-untranslated region (UTR), known to be sufficient to target reporters to the anterior (Macdonald and Struhl, 1988). *cen-bcd-3'UTR* embryos localize *cen* mRNA to the anterior cortex; thus, *cen* mRNA is depleted from centrosomes within the embryonic mid-region. In the absence of local *cen* mRNA, mitotic errors result, centrosomes show aberrant segregation, and genome instability is elevated. This work demonstrates the localization of *cen* mRNA is crucial for the localization of Cen protein to centrosome and essential for normal centrosome function (Ryder et al., 2020).

Although prior work ascribed a role for *cen* mRNA localization to centrosomes, how *cen* mRNA localizes to centrosomes remains unknown. Thus, the main goal of this thesis is to define the mechanism of *cen* mRNA localization to centrosomes. Work from our laboratory and others found *cen* forms ribonucleoprotein granules containing Cen protein. These Cen granules form around the centrosome during the early stages of *Drosophila* embryogenesis in a cell cycle

dependent manner (Ryder et al.,2020). Also, the localization of *cen* mRNA peaks during interphase, specifically at nuclear cycle (NC) of 13, when embryos develop syncytial blastoderm to examine sufficient number of nuclei for quantification (Foe and Alberts, 1983; Ryder et al., 2020). Yet, the regulation of these oscillations is still unknown and how *cen* mRNA localizes to the centrosome is still under investigation. But, we know Cen protein directly binds the centrosomal scaffolding component Centrosomin (Cnn) and organizes the assembly of cleavage furrows during early *Drosophila* embryogenesis (Conduit et al., 2014; Kao and Megraw, 2009). Moreover, the deficiency of *cen* interrupts the organization of the mitotic spindle (Ryder et al., 2020; Kao and Megraw, 2009). *cen* granules also contain a translational regulator, the RNA-binding protein FMRP (Ryder et al., 2020). Loss of *Fmr1*, the gene encoding FMRP, leads to increased recruitment of *cen* mRNA to centrosomes and elevated total Cen protein levels, suggesting FMRP may contribute to *cen* mRNA localization and local protein synthesis (Ryder et al., 2020). Evidence for local translation of Cen is supported by puromycylation proximity ligation assay (Bergalet et al., 2020). To gain a better understanding of *cen* mRNA localization, we aim to decipher the possible *cis*- and *trans*-acting factors that contribute to the formation and localization of *cen* mRNA granules to centrosomes.

To define mechanisms underlying *cen* mRNA localization to centrosomes, we first generated *cen* truncation lines to find the *cis*-acting element that is required for the localization of *cen* mRNA to centrosome. We took advantage of Gibson Assembly technology to construct transgenic *Drosophila* lines to express each truncation (Gibson et al., 2009). The *cen* sequence was divided into two halves, avoiding bisection of predicted protein domains, and an hemagglutinin (HA) tag was added to detect expression via anti-HA antibodies. While we were successful in cloning four different constructs and developed viable lines, our results are

inconclusive as to which half of *cen* contains the sequence sufficient for its localization to the centrosome.

Next, we explored the role of a potential *trans*-acting element of *cen* - Egl. We propose that the directional microtubular network allows *cen* to move in a polarized manner along with motor proteins towards the centrosome. Dynein walks towards the negative end of microtubules. While dynein is an attractive candidate to mediate *cen* mRNA transport to the centrosome, the role of dynein or Egl in *cen* mRNA localization is subject to investigation within our laboratory. To test the contribution of Egl to *cen* mRNA localization, we compared *cen* mRNA localization between wild-type control and strong hypomorphic *egl* mutants. In parallel, we examined a recently defined *egl* mutant with a defective RNA-binding site (*egl**RBD3; Goldman et al., 2021). Our preliminary data suggest *egl**RBD3 mutants negatively affect *cen* mRNA localization to the centrosome, consistent with our working hypothesis. Further examination of *egl* is likely to inform mechanisms required for *cen* mRNA localization to centrosomes important for mitotic fidelity.

Finally, to deepen our understanding of *cen* function during early embryogenesis, we examined the role of local *cen* mRNA and/or protein in supporting centriole duplication and centrosome separation. Embryos null for *cen,* and therefore lacking *cen* mRNA and protein, show normal centriole duplication relative to controls, yet significant delays in centrosome separation (Mehta et al., 2021). Whether local *cen* mRNA or protein impinges on centrosome separation dynamics is a topic for future study.

Results

Toward identifying the cis-element required for cen *mRNA localization to centrosomes*

Prior work suggests the *cen* coding sequence is required for *cen* mRNA localization to centrosomes (Bergalet et al., 2020; Ryder et al., 2020). Nevertheless, the specific sequences within the *cen* coding sequence required for mRNA localization remain elusive. To identify the *cis*-acting element of *cen* mRNA required for centrosomal localization, we designed four different *cen* truncations lines (Figure 1). The *cen* coding sequence is 790 amino acids long, which was truncated into two pieces: an N-terminal and C-terminal truncation. Two versions of each truncation were made by adding an HA tag to either the N-terminus or C-terminus of each sequence. Addition of the HA tag allows for detection of the truncated product via anti-HA antibodies. N- and C-terminal HA tags were generated to control for possible deleterious effects caused by addition of the exogenous tag (e.g., altered protein folding).

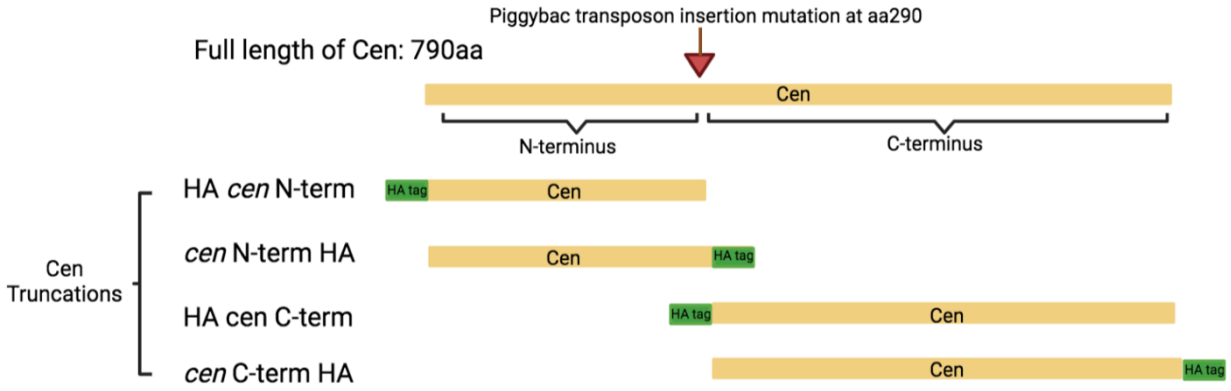

Figure 1. Structure of Cen proteins. Images show four different Cen truncations designs. The amino acids (aa) of where the N-term fragment ends and the C-term fragment begins is labeled.

To generate the truncated Cen products, we used PCR to amplify the pTOPO plasmid backbone and truncated N- and C-terminal *cen* pieces with homologous ends for Gibson ligation.

The expected size of the TOPO backbone is 2.8 kilobases (kb) (Figure 2A), while the *cen* N-terminal truncation is 1.3 kb and the C- terminal truncation is 2.9 kb (Figure 2B). A Gateway destination reaction from pTOPO into a *Drosophila* expression plasmid with a UAS enhancer element and attB sites for site-directed transgenesis was then conducted. The expression plasmids containing the HA tagged truncations were sent to off to a commercial vendor (BestGene, Inc.) for *Drosophila* transgenesis.

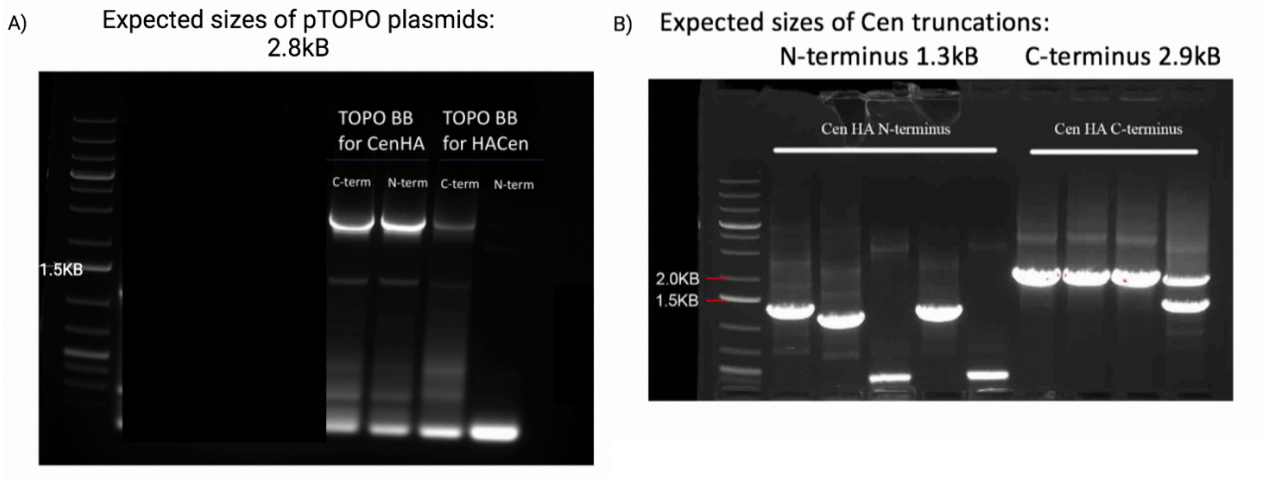

Figure 2. Construction of truncated Cen proteins. (A) Image shows ethidium bromide-stained DNA agarose gel electrophoresis of the pTOPO plasmid backbone (B) Image shows ethidium bromide stained DNA agarose gel electrophoresis of Cen-HA and HA-Cen truncation products following PCR amplification.

Upon receipt of the transgenic *Drosophila* lines, we examined expression of HA-tagged Cen truncation protein products by driving expression of the UAS constructs with a maternally expressed GAL4 driver, *maternal α-Tubulin-GAL4* (*mat>GAL4*). Ovaries from females expressing *mat>GAL4* and *UAS-cen* truncation products were dissected, homogenized, and loaded onto a polyacrylamide gel for protein electrophoresis. The western blot with anti-HA antibodies confirmed expression of the HA-tagged *cen* truncations in all transgenic lines (Figure 3). We further identified the size of each truncation, which came to ~40kD for the N-terminal

and ~70kD for C-terminal Cen truncation protein products. Identity of the Cen truncation products was confirmed using anti-Cen N-terminal and C-terminal antibodies (Kao and Megraw, 2009; Figure 3). Specificity of the bands was demonstrated by including *cen* null protein extracts as a negative control.

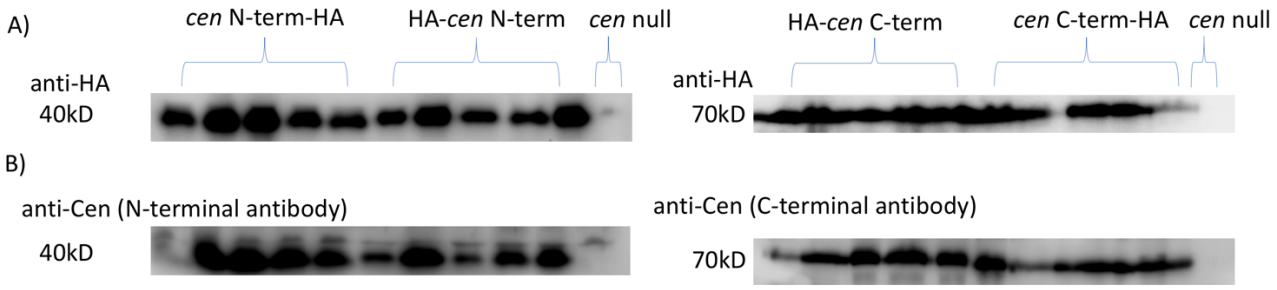

Figure 3 Western blots of truncated protein products. Images show (A) anti-HA and (B) anti-Cen immunoblots of N-terminal and C-terminal Cen truncations

Effect of Cen truncation expression on cen localization to centrosomes

To determine whether either Cen truncation could localize to the centrosome, we performed immunofluorescence (IF) allowing us to examine subcellular localization of truncated Cen proteins. We specifically focused on nuclear cycle (NC) from 11 to 13 embryos, which we previously defined as the peak of *cen* mRNA and Cen protein localization to the centrosome (Ryder et al., 2020). Here, we transgenetically labeled the centrosome by expressing GFP-tagged Cnn (Figure 4, green). Cnn is a conserved component of the centrosome PCM and localizes to the outer edge of the centrosome volume (Lerit et al., 2015; Megraw et al., 1999). Our preliminary data suggests that neither N- nor C- terminal HA-tagged Cen protein truncation was sufficient to localize to the centrosome (Figure 4). There is a possibility that presence of

endogenous Cen interfered the localization of the truncated Cen products, so the expression in the Cen null background will help us to identify functionality and localization of each truncation. Also, each part of the protein, such as N-terminus and C-terminus, may be required for localization. These suggest further design of Cen truncation is needed to identify the zip code as well as enough data points for quantification analysis.

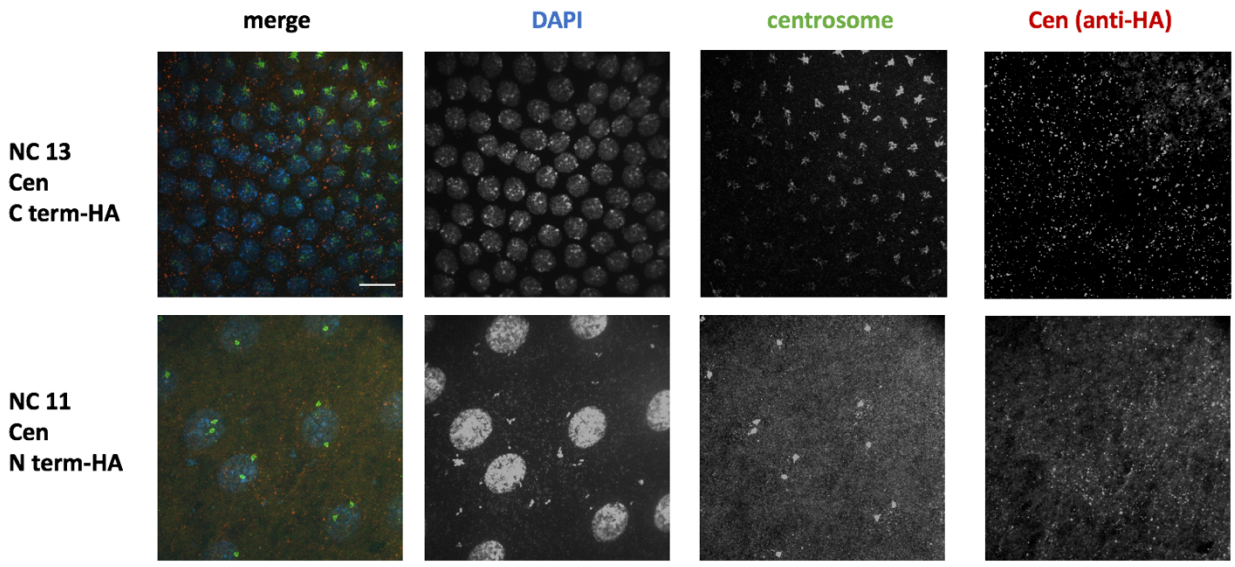

Figure 4 Localization of Cen protein in embryos expressing HA-tagged Cen truncations. These embryos also express endogenous Cen. Cen protein was detected using anti-HA antibodies (red) in embryos expressing GFP-Cnn (green). Nuclei are stained blue with DAPI. For both truncations, Cen fails to localize at the centrosome. Bar = 10 microns.

Toward identifying a* trans*-acting protein required for* cen *mRNA localization to centrosomes

We hypothesize dynein motors facilitate *cen* mRNA localization to centrosomes, a topic currently under examination by postdoctoral fellow, Dr. Hala Zein-Sabatto. Dynein associates with RNA cargoes via the adapter protein Egl (Zein-Sabatto and Lerit, 2021). Therefore, I examined the requirement for Egl in enacting normal *cen* mRNA localization to centrosomes. We attempted to generate transheterozygous *egl* mutant embryos, but failed to recover sufficient embryos. In parallel, we examined a recently defined *egl* mutant, Egl*RBD3, in which eight positively charged residues were converted into alanine (Goldman et al., 2021; Figure 5). This mutation disrupts Egl binding to RNA. We compared *cen* mRNA localization by smFISH in interphase NC 13 wild-type control versus Egl*RBD3 mutant embryos. Our preliminary data suggest *cen* mRNA localization and granule formation around the centrosome is decreased in the Egl*RBD3 mutant relative to the control (Figure 6). These data hint that Egl may play a crucial role in *cen* mRNA localization to centrosome (Figure 7). Further quantification of larger sample sizes is required to establish a role for Egl in *cen* mRNA localization.

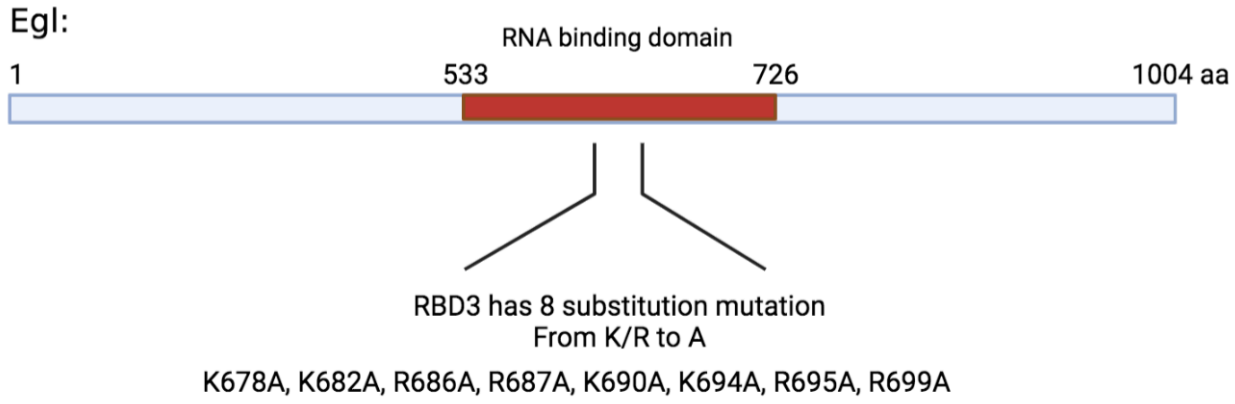

Figure 5 Schematic of the Egl*RBD3 mutation. The amino acid numbers of Egl*RBD3 mutation are labeled. Image adapted from Goldman et al., 2021.

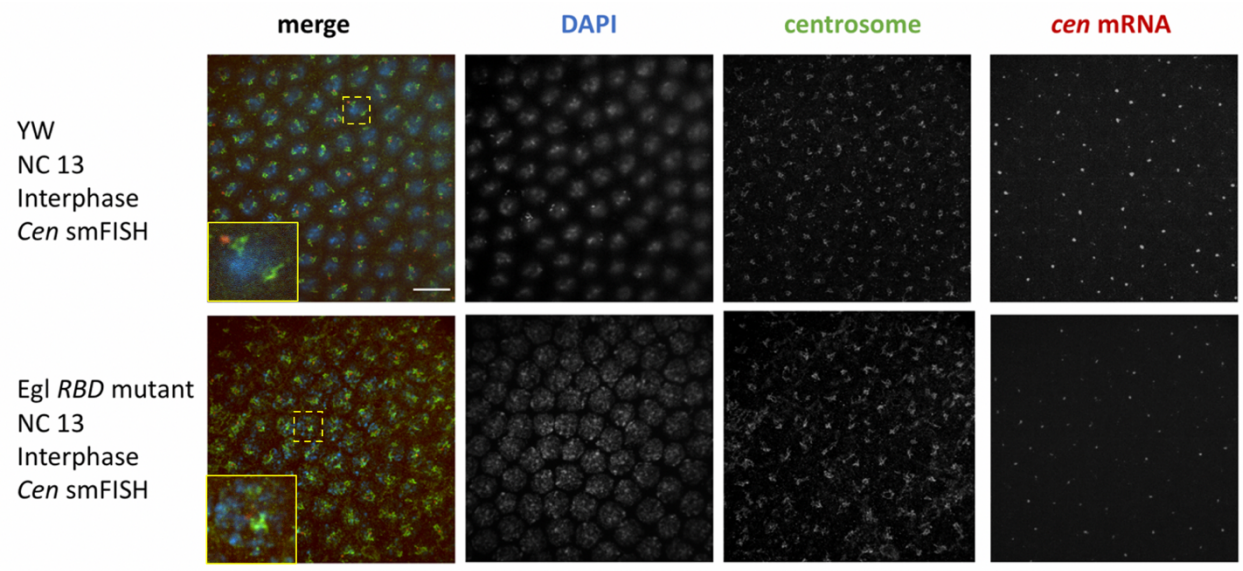

Figure 6. Localization of *cen* mRNA in embryos of Egl RBD mutant and YW. *cen* mRNA was detected using smFISH (red) in embryos expressing GFP-Cnn (green). Nuclei are stained blue with DAPI. Egl RBD mutant shows less formation of *cen* mRNA granules than that of YW. Bar = 10 microns.

cen *is required for centrosome duplication*

To further define the role of *cen* in *Drosophila* embryogenesis, we examined centriole duplication and centrosome separation in *cen* null embryos, which lack *cen* mRNA and protein (Kao and Megraw, 2009). For this study, former postdoctoral fellow Dr. Pearl Ryder conducted live imaging of control and *cen* mutant embryos expressing *GFP-Cnn* to label centrosomes and *Ubi-RFP-PACT* to label the centrioles. The *Ubi-RFP-PACT* transgenic line was a gift from the lab of Dr. Nasser Rusan (NIH), and I extracted genomic DNA and used RFP forward and PACT reverse primers (Table 1) to PCR amplify the tagged fragment of PLP, which was submitted for DNA sequencing. DNA sequencing results confirmed the *Ubi-RFP-PACT* construct was generated from the C-terminal fragment 5 (aa 2,479–3,555) of PLP isoform PF. Detailed analysis of time-lapse recordings, conducted by Dr. Zein-Sabatto and Mr. Dipen Mehta, revealed loss of

cen is associated with delays in centrosome separation at NC 13. In contrast, centriole duplication was unaffected (Mehta et al., 2021).

Discussion

Within most animal cells, the centrosome is the main microtubule organizing center that is required for error-free mitosis. mRNA localizes to defined cellular compartments such as centrosome. Accumulating evidence indicates several mRNAs localize to the centrosome during the cell cycle (Lecuyer et al.,2007; Wilkie and Davis, 2001). A subset of mRNAs, such as *cyclin B* (*cyc B), centrocortin (cen*), and *Pericentrin-like protein* (*plp),* are found to localize near the spindle pole and regulate centrosomal functions (Groisman et al., 2002; Ryder et al., 2020). However, how mRNAs localize to the centrosome remains unclear. This thesis examines the mechanism underlying *cen* mRNA localization to the centrosome, which elevates Cen protein levels, and thereby organizes the assembly of cleavage furrows (Kao and Megraw, 2009; Ryder et al., 2020). Our data supports a model where Egl, an RNA-binding protein and dynein motor adaptor protein, is required for *cen* mRNA localization to the centrosome (Figure 7).

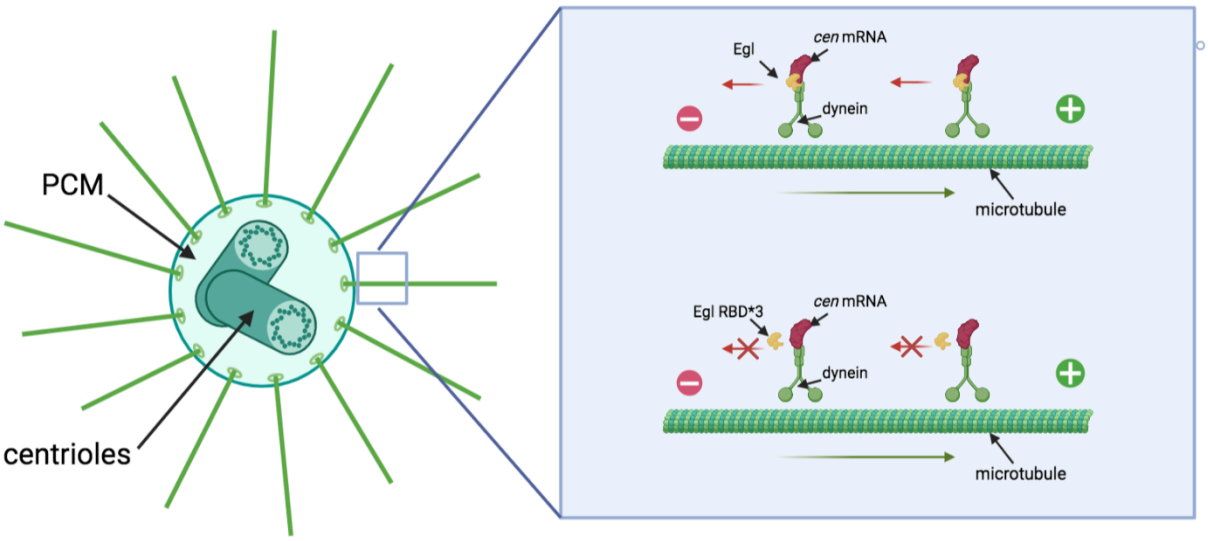

Figure 7 Model for minus-end-directed *cen* mRNA localization with *trans*-acting elements. This cartoon model proposes an interactive relationship between *cen* mRNA with Egl and dynein motor proteins, which allows RNA to traffic towards the centrosome (Chin and Lecuyer, 2017). The schematic shows Egl ensures the localization of *cen* mRNA to centrosome via the dynein motor, and the mutation of Egl results in a failure of *cen* mRNA localization.

To test this model, we wanted to identify the *cis*-acting and *trans*-acting elements required for *cen* mRNA localization. We designed four different Cen truncation products by using *Drosophila* transgenesis (Figure 1). Within each truncation, an HA tag was inserted to detect the truncated products via anti-HA antibodies. We confirmed the expression of the HA-tagged Cen truncations within all transgenic lines (Figure 3). In theory, the Cen truncation that properly localizes to the centrosome would contain the *cis*- element. On the other hand, the lack of localization of a particular Cen truncation to the centrosome would suggest that fragment does not contain the *cis*-element. Using IF, we examined the localization of each Cen truncation relative to the centrosome (Figure 4). Unfortunately, the result of the IF was inconclusive. Our

preliminary data showed neither N- nor C- terminal HA-tagged Cen protein truncation was sufficient to localize to the centrosome. However, it is possible that each part of the protein, such as N-terminus and C-terminus, is required for localization. Therefore, further designs of Cen truncation might be needed. Alternatively, the presence of endogenous Cen protein may interfere with the localization of the truncated products.

Next, we also tested whether Egl is the *trans*-acting elements for *cen* mRNA localization. We tested the contribution of Egl by comparing the localization of *cen* mRNA to the centrosome in wild-type control and hypomorphic Egl mutant embryos. Trans-heterozygous embryos were generated by crossing *egl 3e* and *egl wu50* alleles. First, we transgenetically labeled the centrosome by expressing GFP-tagged Cnn (Figure 3, green). However, the transheterozygous *egl 3e/egl wu50* mutants were not fertile for examination. We attempted to make another transheterozygous line without GFP-tagged Cnn, reasoning the GFP labeled might be affecting the fertility rate. Yet, *egl 3e/egl wu50* without GFP-tagged Cnn still had a low fertility rate. Therefore, we extended the collection time from 0.5-2.5 hours to 0.5-3.5 hours in hopes to collect more fertilized embryos. The collection of is *egl 3e/egl wu50* mutant embryos is still in process.

In parallel to *egl 3e/egl wu50*, we examined Egl*RBD3 mutants, which harbor a defective RNA binding domain. Our preliminary data suggests that less *cen* mRNA granules are formed in Egl*RBD3 relative to the control. Based on our preliminary data, the mutations in the Egl RNA binding domain negatively affected the localization of *cen* mRNA to the centrosome. This data suggests that Egl may be the RNA binding protein of choice for *cen* mRNA to localize at the centrosome, and thereby supports our model (Figure 7). However, in order to reach significance in our data, a larger sample size is needed for quantification. A previous

postdoctoral fellow from our lab developed a Python-based quantification pipeline which we can use to calculate the percentage of total RNA that overlaps with centrosomes (Ryder et al., 2020). Furthermore, a pull-down assay could be conducted to detect whether Cen protein and Egl biochemically interact. A pull-down assay is a technique to test for protein-protein interactions. According to our model, wildtype Egl would pull down Cen protein and mRNA, whereas the Egl*RBD3 mutant with the defective RNA binding domain should not.

We also assessed the centriole duplication and centrosome separation in *cen* null embryos because a RNA localization was postulated to influence centriole duplication (Lawrence and Singer, 1986). Our data indicates that without *cen*, there is a delay in centrosome separation at NC 13, while centriole duplication did not significant change (Mehta et al.,2021). This experiment suggests the presence of *cen* mRNA and Cen protein is dispensable for centriole duplication. However, it remains to be tested if local *cen* mRNA or Cen protein is required for the role in centrosome duplication. How we can uncouple local *cen* mRNA and protein at the centrosome remains a challenge, but one idea we are pursuing in the laboratory is to generate a non-translatable version of *cen* mRNA and to test whether known Cen functions (e.g., centrosome separation, mitotic fidelity) are altered. If the protein is required, then we expect these processes to look like the null. If the process was not altered, then we could directly attribute functions to the mRNA. Dissecting distinct roles requires an in-depth analysis. Presently, this functional characterization is progressing at a far slower pace than the discovery of mRNAs localized to the centrosome (Zein-Sabatto and Lerit., 2021)

Understanding the localization mechanism of *cen* mRNA to the centrosome can inform our understanding of subcellular localization of various other mRNAs. Localization of mRNA at the centrosome is evolutionarily conserved (Groisman et al., 2000; Lecuyer et al., 2007; Zein-

Sabatto and Lerit, 2021). The model that we are suggesting (Figure 7) uses active transport by means of a minus-end directed motor protein, dynein. Dynein is a common vehicle that transports various cargos across the microtubule network. Failure of this universal, yet tightly regulated mode of subcellular transport leads to many cellular dysfunctions. Specifically, mislocalization of centrosome localizing mRNAs, such as *cen,* causes centrosomal dysfunctions such as cell cycle abnormalities, disrupted microtubule organization, and genomic instability (Ryder et al., 2020). Eventually, these cellular abnormalities underly larger developmental problem, such as intellectual disabilities and diseases such as cancer (Conduit et al., 2015).

Material and Experimental Procedures

Fly Stocks

The following *Drosophila* lines were used in this study: yw^{1118} were used as a control; *cen* C-terminus HA truncation and *cen* N-terminus HA truncation (this study); the hypomorphic allele *egl 3e* (Pelegri,1994; Mach and Lehmann, 1997) and the null allele *egl wu50* (Schupbach and Wieschaus, 1991; Mach and Lehmann, 1997) were used with GFP-Cnn*; egl* shRNA-1 with *Egl_RBD3* was tagged on the C-terminus with 3xFLAG (Goldman et al., 2021) and it was driven by maternal alpha tubulin-GAL4. Flies were raised and maintained at 25°C in a light- and temperature-controlled chamber.

Construction of *cen* truncations

To generate HA-tagged *cen* truncations, the pEntr/TOPO plasmid backbone was PCR amplified. Then, inserts comprising the Cen coding sequence were also amplified to create homologous, partially overlapping "sticky ends." The HA-tag was incorporated as pre-synthesized oligomers. Primers are listed in Table 1. TOPO backbones and inserts were ligated with Gibson Assembly using an 1:1:2 molar ratio of plasmid:Cen CDS:HA tag. Sequences were verified by DNA sequencing, and they were recombined into the pPWattB destination vector using the Gateway cloning system. *Drosophila* transgenesis was carried out by BestGene, Inc. Five independent lines were obtained per insertion into the third chromosome by PhiC31 integrase-mediated transgenesis.

TABLE1: List of primers used in this study.

Primer	Description
M13Forward M13Reverse	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC To check colony PCR of *cen* truncations line
RFP_Forward PACT_reverse	ACCATCGTGGAACAGTACGAG CCAGATGCGTCCAATGTATTT To extract genomic DNA and PCR amplify the tagged fragment of PLP
C-term HA tag N-term HA tag	GGCGGCAGCGGTGGAAGTGGTGGTAGTGGAGGAAGTTACCCATACGATGTTC CTGACTATGCGGGCTATCCCTATGACGTCCCGGACTATGCAGGATCCTATCCA TATGACGTTCCAGATTACGCTTAA ATGTACCCATACGATGTTCCTGACTATGCGGGCTATCCCTATGACGTCCCGGA CTATGCAGGATCCTATCCATATGACGTTCCAGATTACGCTGGCGGCAGCGGTG GAAGTGGTGGTAGTGGAGGAAGT To insert a HA tag to each N- and C-terminus.
pTOPO cen HA Forward pTOPO N-term Cen HA Reverse pTOPO C-term Cen HA Reverse	CCTATCCATATGACGTTCCAGATTACGCTTAAAAGGGTGGGCGCG CCGTGATTGGATTCCTCCATGGTGAAGGGGGCGGC GTGTTGCTTTCGTTAATCATGGTGAAGGGGGCGGC To create TOPO backbone for Cen HA
Cen HA C-term Forward Cen HA C-term Reverse	CCGCGGCCGCCCCCTTCACCATGATTAACGAAAGCAACACCAATATGGA CCACTTCCACCGCTGCCGCCCTTTTGACGAAACTGATGATGATGACTCTTG To create Cen HA C-terminus truncation
Cen HA N-term Forward Cen HA N-term Reverse	CCGCGGCCGCCCCCTTCACCATGGAGGAATCCAATCACGGTTC CCACTTCCACCGCTGCCGCCATCCCTCAGGCAGCGACT To create Cen HA N-terminus truncation
pTOPO HA Cen Reverse pTOPO HA Cen N-term Forward pTOPO HA Cen C-term Forward	CGCATAGTCAGGAACATCGTATGGGTACATGGTGAAGGGGGCGGC GTAGTCGCTGCCTGAGGGATTAAAAGGGTGGGCGCGC CATCAGTTTCGTCAAAAGTAAAAGGGTGGGCGCGC To create TOPO backbone for HA Cen
HA Cen C-term Forward	GGTGGAAGTGGTGGTAGTGGAGGAAGTATTAACGAAAGCAACACCAAT ATGGATG

HA Cen C-term Reverse	GGGTCGGCGCGCCCACCCTTTTACTTTTGACGAAACTGATGATGATGAC To create HA Cen C-terminus truncation
HA Cen N-term Forward HA Cen N-term Reverse	GGAAGTGGTGGTAGTGGAGGAAGTGAGGAATCCAATCACGGTTCGG TCGGCGCGCCCACCCTTTTAATCCCTCAGGCAGCGACT To create HA Cen N-terminus truncation

Western Blotting

Females were fattened on yeast for one day before dissection. Ovaries were dissected and fixed in 1x PBST and 5x SDS loading dye and boiled at 95°C for 5 min. The samples were run on an SDS-PAGE gel and transferred into nitrocellulose membrane by wet transfer. The membrane was blocked in 5% dry milk solution in TBST (Tris-based saline with 0.05% Tween-20). Then, the membrane was incubated in TBST with diluted primary antibodies: anti-HA (Cell Signaling mAb rabbit (C29F4); 1:1,000), anti-Cen (both N- and C- terminal antibodies were gifted from Tim Megraw; 1:500) overnight at 4°C. Next day, the membrane was incubated in TBST with diluted secondary antibodies (HRP Conjugated; 1:2,500) for 1 hour. The sample was again washed several times in TBST, and the blots were imaged on a ChemiDoc (Bio-Rad) gel imager.

Fixed Embryos with Immunofluorescence

0.5-2.5 hour embryos were collected and fixed in a 1:4 solution of 4% paraformaldehyde/heptane for 20 min and devitellinized in methanol. Samples were rehydrated into PBST stepwise in a methanol: PBST series (3:7, 1:1, 7:3), then washed in PBST and stained with smFISH probes or antibodies. For smFISH, embryos were incubated with probes at 37°C water bath overnight. Next day, probes were removed from embryos by using pre-warmed WB* buffer. Then, samples were blocked in the FISH-BBT solution (PBST with 0.1% BSA) for 2 hours. Embryos were

incubated with primary antibody in FISH-BBT overnight at 4°C. Next day, embryos were washed at room temperature in FISH-BBT for 1 hour. Embryos were incubated in FISH-BBT with diluted secondary antibody and DAPI for 2 hours. Embryos then were washed with PBST several times and mounted in a Vectashield medium. Rabbit anti-Cnn 1:3500 was used as the primary antibody; guinea pig anti-rabbit 488 1:500 was used as the secondary antibody. For Cen truncations, embryos were collected and fixed as described above, blocked with Image-iT FX singal enhancer (Invitrogen) for 1 hour at the room temperature, then incubated with rabbit anti-Cnn (1:1,000) and mouse anti-HA (Invitrogen 5B1D10; 1:500) as primary antibodies overnight at 4°C. The next day, embryos were washed with PBST, incubated with DAPI (1:1,000), and mounted with Vectashield.

Detection of RNA through smFISH

All smFISH protocols were performed with RNase-free solutions. Collected 0.5-2.5 hours embryos were rehydrated in 100% methanol (3:7, 1:1, 7:3) and PBST and then washed in WB* buffer and HB* buffer. Then, embryos were incubated at 37°C water bath in *cen* probe diluted 1:50 in HB* buffer. smFISH probes were designed to bind to complementary RNA to make specific RNA fluorescent under the microscope. After the overnight water bath incubation, embryos are washed repeatedly in pre-warmed WB* buffer (15 minutes for three times). Then, embryos were stained with DAPI for 30 minutes, washed with PBST three times for five minutes each, and mounted with Vectashield mounting medium. Slides were stored at 4°C avoiding light and imaged within three days.

Microscopy

Images were captured on a Nikon Ti-E system fitted with Yokagawa CSU-X1 spinning disk head, Hamamatsu Orca Flash 4.0 v2 digital CMOS camera, Nikon LU-N4 solid state laser launch (15 mW 405, 488, 561, and 647 nm) using the following objectives: ×100, 1.49 NA Apo TIRF oil immersion objective. Images were acquired using a Vectashield medium.

Image Analysis

Images were adjusted using the FIJI program and Microsoft PowerPoint to separate or merge the channel, create the maximum intensity projection, adjust the brightness and contrast, and crop regions of interest.

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