Molecular Details and Functional Analysis of RNA Binding by ESCRT-II

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Molecular details and functional analysis of RNA binding by ESCRT-II

A dissertation presented

by

Amy Beth Emerman

to

The Division of Medical Sciences

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Molecular details and functional analysis of RNA binding by ESCRT-II

Abstract

Many RNAs show distinct localization patterns in cells with enrichment at particular subcellular sites or organelles. RNA localization is a highly conserved process that both spatially and temporally controls gene expression. A common mechanism to selectively sort RNAs within the cell involves recognition of cis-acting sequences on the RNA by trans-acting RNA-binding proteins. Recently, the ESCRT-II complex was identified as a novel trans-acting factor required for the localization of bicoid mRNA in Drosophila oocytes. ESCRT-II was previously uncharacterized as an RNA-binding complex but has a well-established role in multivesicular body formation and receptor downregulation. Recent studies have revealed links between endosomes and RNA regulatory pathways, and the dual roles of ESCRT-II in both cellular processes suggest that it will be an important factor to better understand as an RNA-binding complex. However, bicoid is the only identified direct RNA target of ESCRT-II, and whether ESCRT-II’s role in RNA localization is conserved in other organisms is unclear.

Here we report that the role of ESCRT-II in RNA regulation is conserved in Xenopus eggs. We found that ESCRT-II interacts with hundreds of RNAs in Xenopus eggs, and we characterized the molecular details of this interaction. Using a UV-crosslinking approach, we show that ESCRT-II binds directly to RNA through the subunit Vps25. Furthermore, by performing CLIP-seq, we found that ESCRT-II recognizes a polypurine motif. Selective binding of the polypurine motif through Vps25 can be recapitulated in vitro by multiple binding assays using purified components. Furthermore, ESCRT-II interacts with a subset of RNAs that are
enriched on the mitotic spindle, and we provide preliminary evidence that ESCRT-II may be involved in localizing RNAs to the mitotic spindle. Consistent with previous reports, we found that ESCRT-II localizes to the centrosome in *Xenopus* tissue culture cells and to exogenous centrosomes added to egg extract. Our results suggest that the role of ESCRT-II in RNA regulation is conserved and shed light on an unexpected link between the cellular systems that control endosomal sorting and RNA localization.
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For my parents,
for their unwavering love and support
RNA localization as a mechanism of gene regulation

Individual cells in a multicellular organism can display drastically different cell fates despite the fact that they contain the same genome, indicating that these distinct cell types arise from differential usage of the same genetic information. This differential gene expression can be achieved by a variety of mechanisms at both the transcriptional and translational level. A major mechanism that the cell utilizes to control gene expression is through the regulation of mRNAs. The physical separation of transcription and translation of mRNAs in eukaryotes provides several steps that can be regulated to fine tune gene expression. Consequently, an mRNA is regulated throughout its lifespan; for instance, at pre-processing in the nucleus (capping, splicing, and addition of the poly(A) tail), nuclear export, localization in the cytoplasm, translation, and degradation.

The localization of an RNA is a particularly important regulatory step that serves to both spatially and temporally control gene expression. A localized RNA is any RNA that does not show uniform distribution in the cytosol, but rather a concentration at a particular region of the cell or subcellular structure. When coupled with translational regulation, mRNA localization restricts the time and region in which a protein is expressed (reviewed in Besse and Ephrussi, 2008). Early hints that RNAs can show subcellular localization patterns were found in germ cells, where many maternal RNAs localize to a defined region of an oocyte to be asymmetrically segregated in the early cell divisions that follow fertilization (reviewed in Medioni et al., 2012). Early experiments that aimed to better understand the cytoplasmic maternal factors that influence cell fate in developing embryos revealed an uneven distribution of poly(A)+ RNA in the ascidian...
oocyte (Jeffery and Capco, 1978) as well as oocytes from a variety of other organisms (reviewed in Jeffery, 1989). Subsequent studies revealed that some mRNAs, specifically β-actin mRNA, are unevenly distributed in the ascidian oocyte, while other mRNAs, including those that code for histones, show an even distribution (Jeffery et al., 1983).

Germ cells have been a particularly useful model system to understand the importance of localized RNAs. In *Xenopus laevis* oocytes, small subsets of RNAs are localized to either the animal pole or the vegetal pole, and these RNAs are asymmetrically segregated in the early embryo (Rebagliati et al., 1985) (Figure 1.1A). Currently, more than twenty RNAs have been identified that are enriched at the vegetal pole of the *Xenopus* oocyte, and at least sixteen RNAs are enriched at the animal pole (reviewed in King et al., 2005). Many of the localized mRNAs code for factors that are involved in cell fate determination in the developing embryo. For example, VegT is a vegetally localized mRNA that codes for a T-box transcription factor and influences mesoderm patterning in the embryo (Zhang and King, 1996). Another vegetally localized RNA, Vg1, codes for a TGFβ-related growth factor and is involved in the induction of the dorsal mesoderm (Birsoy et al., 2006; Dale et al., 1993; Kessler and Melton, 1995; Thomsen and Melton, 1993). Similarly, in *Drosophila* oocytes, several maternal mRNAs show distinct localization patterns (Figure 1.1B). Among these mRNAs are *bicoid, nanos, oskar,* and *gurken,* which were originally identified in genetic screens as maternal effect genes involved in establishing anteroposterior or dorsoventral body patterning in *Drosophila* (reviewed in Govind and Steward, 1991; Nusslein-Volhard et al., 1987), and the localization of these mRNAs is critical for this role (Berleth et al., 1988; Driever et al., 1990; Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992; Neuman-Silberberg and Schupbach, 1993, 1994; Wang and Lehmann, 1991). For example, the anterior localization of *bicoid* mRNA
Figure 1.1: Examples of localized RNAs in diverse organisms and cell types.
A) bicoid (bcd), gurken (grk), and oskar (osk) mRNAs localize to distinct regions of *Drosophila* oocytes. B) Vg1 and VegT mRNAs localize to the vegetal pole of *Xenopus* oocytes. C) β–actin mRNA localizes to the leading edge of migrating fibroblasts. D) CamKIIa mRNA localizes to the dendrites of neurons. E) *ASH1* mRNA localizes to the daughter cell during cell division in budding yeast. Reprinted from (Cody et al., 2013) with permission from Wiley Periodicals, Inc. © 2013.

establishes a gradient of bicoid protein emanating from the anterior pole of the *Drosophila* embryo that influences head and thorax patterning (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988; Driever et al., 1990), while the posterior localization of *nanos* mRNA establishes an opposing gradient of nanos protein to influence abdomen development (Gavis and Lehmann, 1992; Wang et al., 1994; Wang and Lehmann, 1991). In both *Xenopus* and *Drosophila*
oocytes, injection of localized RNAs into regions of the cell where they are typically absent leads to defects in development (for example, Dale et al., 1993; Driever et al., 1990; Kessler and Melton, 1995; Thomsen and Melton, 1993; Zhang and King, 1996), highlighting the importance of RNA localization and spatial restriction of protein expression in oocytes.

While the earliest examples of localized RNAs were in germ cells, it is now recognized that RNA localization likely occurs in all cells types. In migrating cells, such as chicken embryo fibroblasts, \( \beta \)-actin mRNA localizes to the leading edge of the cell (Lawrence and Singer, 1986) (Figure 1.1C). Disruption of \( \beta \)-actin mRNA localization or inhibition of new translation dampens the ability of the cell to migrate, suggesting that localized translation of \( \beta \)-actin mRNA is important for cell migration (Kislauskis et al., 1997). Indeed, colocalization of \( \beta \)-actin mRNA, actively translating \( \beta \)-actin protein, and mature \( \beta \)-actin protein has been observed at the leading edge of migrating cells, providing further evidence that \( \beta \)-actin mRNA localization occurs to locally translate \( \beta \)-actin protein for cell migration (Rodriguez et al., 2006).

RNA localization is especially important in large and highly polarized cells such as neurons, in which distinct subcellular domains perform dramatically different functions (Figure 1.1D). For example, \( \beta \)-actin mRNA becomes asymmetrically localized at the growth cones of neurons in response to external cues in order to directionally guide growth through localized translation (Leung et al., 2006; Yao et al., 2006; Zhang et al., 2001). Several other axonal and dendritically localized mRNAs have been well studied in the neurons of a variety of organisms, including CamKII\( \alpha \), MAP2, and tau mRNAs (reviewed in Bassell and Singer, 2001), and additional genome-wide sequencing and microarray analyses from isolated axons and dendrites have revealed that hundreds of mRNAs are specifically enriched in neuronal processes (reviewed
in Medioni et al., 2012). The long distances that the ends of neuronal processes can be from the cell body highlight the importance of localized translation in this system; by localizing an mRNA to distal regions where its protein product is needed, the cell can rapidly and efficiently produce a translational output in response to local stimuli.

Even single cell organisms use RNA localization as a mechanism of gene regulation. Organisms as simple as bacteria have examples of localized mRNAs (Nevo-Dinur et al., 2011). In addition, in budding yeast, more than 30 RNAs segregate asymmetrically to the daughter cell during cell division (Aronov et al., 2007; Shepard et al., 2003). One of the best-studied examples of these is ASH1 mRNA (Long et al., 1997; Takizawa et al., 1997) (Figure 1.1E). Following cell division, the protein product of ASH1(Ash1p) suppresses the expression of the HO endonuclease gene in the daughter cell, which when expressed in the mother cell (due to the absence of Ash1p) leads to mating type switching (Bobola et al., 1996; Sil and Herskowitz, 1996). Thus, the act of localizing an mRNA in yeast leads to differential gene expression and cell fate among the mother and daughter cell.

The examples of localized RNAs discussed so far highlight some of the biological purposes for this phenomenon. Localized RNAs in Xenopus oocytes and yeast demonstrate that some RNAs unevenly distribute in cells to be positioned for asymmetric segregation following cell division. The examples in neurons and migrating cells demonstrate that some mRNAs are positioned to undergo localized translation, which allows for efficient and rapid targeting of proteins to a region. Finally, in some cases mRNAs localize to prevent the expression of a protein in regions of the cell where such expression would be detrimental. For example, translation of myelin basic protein (MBP) is restricted to distal processes of oligodendrocytes where MBP compacts membranes to form myelin sheaths (Colman et al., 1982), and
misexpression of MBP in the cell body among essential membranous organelles would likely be deleterious to the cell (Lyons et al., 2009). The implications of RNA localization pathways in a variety of diseases, including neurodegenerative disorders and cancer, highlight the biological importance of this mechanism of regulation (reviewed in Cody et al., 2013).

**RNA enrichment on organelles and subcellular structures**

While the classical examples of localized RNAs are in large or highly polarized cells, more recent studies have demonstrated that RNAs are unevenly distributed in most cell types through selective associations with various organelles or subcellular structures. In fact, the majority of transcripts are likely not uniformly distributed in the cytoplasm, as exemplified by a recent high-throughput FISH study in *Drosophila* embryos that showed that out of approximately 3000 transcripts analyzed, 71 percent show some pattern of subcellular localization (Lecuyer et al., 2007).

The mitotic spindle exemplifies a subcellular structure to which many mRNAs localize. In most cell types, the mitotic spindle is primarily composed of centrosomes, microtubules, and chromatin. Centrosomes associate with a unique sets of mRNAs in a variety of organisms, including snails (Kingsley et al., 2007; Lambert and Nagy, 2002), surf clams (Alliegro and Alliegro, 2008; Alliegro et al., 2006), and flies (Lecuyer et al., 2007; Raff et al., 1990). Furthermore, the localization of several RNAs (such as cyclinB1, Xbub3, xkid, and tpx2) to the mitotic spindle was revealed by *in situ* hybridization in *Xenopus* oocytes (Eliscovich et al., 2008; Groisman et al., 2000). The prevalence of spindle-localized RNAs was further ascertained by genome-wide studies in which taxol-stabilized microtubules from meiotic *Xenopus* egg extracts or mitotic HeLa cell lysates were isolated as a proxy for the spindle and the associated RNAs...
were sequenced, revealing hundreds of microtubule-associated RNAs (Blower et al., 2007; Sharp et al., 2011). The microtubule-associated mRNAs encode proteins associated with the cell cycle, cytoskeleton/centrosomes, and chromosome structure/segregation. These data suggest that localized translation may be one reason that these RNAs target to the spindle, as seen for cyclinB1, Xbub3, and xkid in *Xenopus* oocytes (Eliscovich et al., 2008; Groisman et al., 2000). The localization of these RNAs, however, is translation-independent (Blower et al., 2007).

Alternatively, some RNAs target to the mitotic spindle in order to be asymmetrically sorted during cell division (Kingsley et al., 2007; Lambert and Nagy, 2002), or to play a noncoding role in spindle formation (Blower et al., 2005; Ideue et al., 2014; Jambhekar et al., 2014; Rosic et al., 2014).

The endoplasmic reticulum (ER) represents another site of abundant RNA localization. The examples of localized RNAs discussed so far involve RNA localization prior to and independent of protein translation. An additional mechanism to localize mRNAs to subcellular structures is to localize the mRNA during translation through recognition of the nascent peptide chain. Secreted and transmembrane proteins contain a signal sequence that is recognized during translation by signal recognition particle (SRP), which directs the nascent polypeptide, ribosome and associated mRNA to the ER (Walter and Lingappa, 1986). However, several pieces of evidence suggest that there are mechanisms to target mRNAs to the ER independently of translation. Early cell fractionation experiments found a large overlap between mRNAs that associate with free- versus membrane-bound ribosomes (Mueckler and Pitot, 1981), and more recent studies confirmed that mRNAs that code for both secretory and cytoplasmic proteins associate with membranes (Diehn et al., 2000; Lerner et al., 2003). Experiments in which EDTA is used to dissociate ribosomes, SRP is knocked down, or mutations are made to inhibit
translation of particular mRNAs maintain the ER localization of several mRNAs encoding ER-
resident proteins, suggesting the existence of an alternate pathway for mRNA localization to the
ER (Chen et al., 2011; Cui et al., 2012; Pyhtila et al., 2008). One protein, p180, has been
identified that is capable of targeting mRNAs to the ER in a translation-independent manner (Cui
et al., 2012). The redundancy of localizing mRNAs to the ER by both mRNA- and peptide-based
mechanisms could exist in order to make the process of translation at the ER more efficient or to
ensure that essential ER-resident proteins can get translocated into the ER when the SRP system
is overloaded.

A similar combination of translation-dependent and –independent RNA targeting events
occurs for RNA localization to the mitochondria, where hundreds of mRNAs transcribed by the
nuclear genome localize (Gadir et al., 2011; Garcia et al., 2007; Marc et al., 2002; Saint-Georges
et al., 2008). Puf3p is responsible for localizing a subset of the mitochondria-localized RNAs
through direct binding to the RNA (Gadir et al., 2011; Saint-Georges et al., 2008). While it has
generally been thought that proteins are posttranslationally targeted to the mitochondria, these
findings challenge that dogma and suggest that for at least some mitochondrial proteins, a
combination of targeting mechanisms exist.

Thus, mRNA localization is widespread throughout biology. In highly polarized cells,
RNAs localize to specialized regions of the cell, while in most cells, RNAs are selectively sorted
to organelles and subcellular structures. While the biological importance of RNA localization in
highly polarized cells can be clearly demonstrated, the effect of RNA sorting in additional cell
types may be more subtle. Such events may occur for efficient targeting of nascent proteins to
the sites where they are needed, to promote asymmetric sorting during cell division, or to target
noncoding or structural RNAs to particular sites. While the study of RNA sorting to organelles
and subcellular structures is relatively new, its prominence suggests that these events are likely
to have important impacts on cellular function and organization.

Mechanisms to localize RNAs

In many cases, RNAs are localized in the cell through active transport on the
cytoskeleton. For example, inhibition of microtubule polymerization disrupts the localization of
Vg1 to the vegetal cortex of *Xenopus* oocytes (Yisraeli et al., 1990), as well as the localization of
*bicoid* to the anterior pole of *Drosophila* oocytes (Pokrywka and Stephenson, 1991). Likewise,
the actin cytoskeleton is required for the localization of *ASH1* mRNA in yeast (Long et al., 1997;
Takizawa et al., 1997) and β-actin mRNA in fibroblasts (Sundell and Singer, 1991). Motor
proteins such as dynein, kinesins, and myosins are required for the localization of many mRNAs;
for example, Vg1 requires dynein and multiple kinesins (Betley et al., 2004; Gagnon et al., 2013;
Messitt et al., 2008), *ASH1* requires myosin (Long et al., 1997; Takizawa et al., 1997), and *bicoid*
requires dynein (Duncan and Warrior, 2002; Januschke et al., 2002; Schnorrer et al., 2000) for
localization. While directed, active transport is the best-studied mechanism to localize an RNA,
additional mechanisms exist, including localization by diffusion and entrapment and localized
protection from degradation (reviewed in Besse and Ephrussi, 2008).

Cis-acting sequences and trans-acting factors

In order to achieve selective partitioning of RNAs within the cell, mechanisms must exist
to identify which RNAs need to be transported to each location. One of the best-studied
mechanisms to localize an RNA is through recognition of a *cis*-acting sequence on the RNA,
often in the 3’ UTR, by a *trans*-acting factor (reviewed in Martin and Ephrussi, 2009). In many
cases, trans-acting factors connect RNAs to active transport machinery in order to promote localization. mRNAs that are undergoing localization are typically translationally repressed during transport, and several trans-acting factors have dual roles in both RNA localization and translational repression (reviewed in Besse and Ephrussi, 2008).

Many cis-acting sequences that direct RNA localization have been identified through the use of genetic deletions or exogenously expressed or injected RNAs that harbor deletions. For example, the cis-acting sequence, or “zipcode”, required for β-actin mRNA localization in fibroblasts was identified through experiments in which portions of the β-actin 3’UTR were fused to β-galactosidase and localization of β-galactosidase activity was monitored (Kislauskis et al., 1994). These experiments mapped the β-actin zipcode to an evolutionarily conserved 54 nucleotide (nt) region (Kislauskis et al., 1994). This same zipcode is responsible for β-actin mRNA localization to the growth cones of neurons (Zhang et al., 2001), demonstrating that cis-acting sequences can be conserved across different cell types. Subsequent experiments identified ZBP-1 as the trans-acting factor that recognizes this 54 nt region and directs β-actin mRNA localization (Farina et al., 2003; Ross et al., 1997; Zhang et al., 2001).

Additional conserved cis-acting sequences were found for the vegetal localization of RNAs in Xenopus oocytes. The cis-acting localization element that directs Vg1 mRNA to the vegetal cortex was originally mapped to a 340 nt sequence in the Vg1 3’ UTR (Mowry and Melton, 1992) and was further mapped to clusters of redundant, 5-6 nt motifs within these 340 nts (Deshler et al., 1997; Gautreau et al., 1997; Lewis et al., 2004). UV-crosslinking experiments identified Vg1RBP/Vera and hnRNP1 as trans-acting factors involved in the localization of Vg1 (Cote et al., 1999; Deshler et al., 1998; Deshler et al., 1997; Havin et al., 1998). Similar motifs were identified to direct VegT mRNA localization through interactions with Vg1RBP/Vera and
hnRNP1 (Bubunenko et al., 2002; Kwon et al., 2002). Interestingly, Vg1RBP /Vera is highly homologous to ZBP-1 (Deshler et al., 1998; Havin et al., 1998). These findings further highlight the conservation of cis-acting sequences and trans-acting factors in RNA localization pathways.

Cis-acting sequences can be both sequence- and structure-dependent. The localization element that directs bicoid mRNA to the anterior pole of Drosophila oocytes was originally mapped to 625 nts, and this localization element folds into a complex secondary structure (Brunel and Ehresmann, 2004; Macdonald and Struhl, 1988). Later, shorter regions within the 625 nt localization element of bicoid were identified to direct specific stages of its localization (Macdonald and Kerr, 1997; Macdonald et al., 1993). Compensatory mutations that alter the primary sequence but not the structure of the localization element maintain bicoid localization (Macdonald and Kerr, 1998), and bicoid transcripts dimerize to recruit Staufen (Ferrandon et al., 1997), a conserved trans-acting factor involved in bicoid localization (Ferrandon et al., 1994). Thus, in some cases, localization elements can be quite complex and can involve secondary structure and as well as multiple domains.

Many additional cis-acting sequences have been identified for localized RNAs in a variety of systems, including neurons, budding yeast, and others (reviewed in Jambhekar and Derisi, 2007). The development of CLIP-Seq, in which footprints of RNA-binding proteins are identified by UV crosslinking followed by RNase treatment of unprotected RNAs (Ule et al., 2005; Ule et al., 2003), has already and will likely continue to accelerate the identification of additional cis-acting sequences required for RNA localization. For example, CLIP-seq of FMRP, a trans-acting factor involved in RNA localization in neurons, revealed hundreds of direct targets of FMRP and two novel RNA recognition elements (Ascano et al., 2012; Darnell et al., 2011). Furthermore, CLIP-seq of the neuron specific RNA-binding protein, Nova, identified GIRK2
mRNA as a direct target of Nova, and the GIRK2 CLIP-tag is necessary for Nova-dependent localization of GIRK2 mRNA to dendrites (Racca et al., 2010).

In many cases, an RNA becomes loaded with trans-acting factors in the nucleus that can either direct localization of the RNA in the cytoplasm or can recruit additional factors in the cytoplasm that are responsible for localization. Likewise, processing events in the nucleus, such as splicing, can influence RNA localization in the cytoplasm. For example, the localization of oskar mRNA requires splicing of the first intron of the pre-mRNA as well as members of the exon junction complex (Hachet and Ephrussi, 2004; reviewed in Martin and Ephrussi, 2009). In the Xenopus oocyte, several trans-acting factors associate with Vg1 mRNA in the nucleus, and the Vg1 ribonucleoprotein complex (RNP) is remodeled following export into the cytoplasm (Kress et al., 2004; Lewis et al., 2008). A variety of additional nuclear acquired RNPs have been described, highlighting the prevalence of this phenomenon (reviewed in Giorgi and Moore, 2007).

**Identification of ESCRT-II as a trans-acting factor for RNA localization**

In an effort to identify additional trans-acting factors involved in RNA localization, Irion and colleagues performed an unbiased genetic screen in Drosophila in which bicoid mRNA localization was monitored in germline mutants (Irion and St Johnston, 2007). One complementation group was identified that belonged to a member of the ESCRT-II complex. ESCRT-II is a four subunit complex composed of two copies of Vps25 and one copy each of Vps36 and Vps22 (Babst et al., 2002b; Hierro et al., 2004; Im and Hurley, 2008; Teo et al., 2004). Prior to this discovery, ESCRT-II was not known to be involved in RNA regulation, and instead is well characterized as one of several complexes involved in multivesicular body (MVB)
formation and receptor downregulation (more thoroughly described in the following section).

Mutations in any member of the ESCRT-II complex impair bicoid localization (Irion and St Johnston, 2007). However, mutations in other members of the ESCRT pathway have no effect on bicoid localization, suggesting that the role of ESCRT-II in RNA trafficking is independent of its role in membrane trafficking. The authors found that the GLUE domain of Vps36, a subunit of ESCRT-II, is involved in directly binding to bicoid mRNA. To date, no other direct RNA targets of ESCRT-II have been discovered, although a role of ESCRT-II in HIV viral RNA trafficking has been suggested (Ghoujal et al., 2012). Of note, ESCRT-II has been suggested to interact with the dynein-dynactin complex indirectly through an interaction with the Rab7-interacting lysosomal protein, RILP (Jordens et al., 2001; Progida et al., 2006; Wang and Hong, 2006), suggesting the possibility that ESCRT-II could link RNAs to directed trafficking mechanisms. As discussed in the following sections, the dual roles in RNA binding and membrane trafficking make ESCRT-II an important complex to further understand as an RNA-binding factor.

**Functions of ESCRT complexes**

While little is known about ESCRT-II in RNA regulation, extensive studies have uncovered the roles of ESCRT-II on endosomes. ESCRT-II and the additional ESCRT complexes were initially discovered through an analysis of a collection of yeast mutants that had defects in protein sorting to the vacuole (the yeast equivalent of the lysosome), identifying a large set of vacuolar protein sorting (Vps) mutants (Raymond et al., 1992). A subset of these mutants had defects in membrane trafficking that led to the “Class E” phenotype, in which the morphology of the endosome is altered to appear as stacked cisternae and the endosome accumulates golgi, endocytic, and vacuolar proteins (Piper et al., 1995; Raymond et al., 1992;
Rieder et al., 1996). Additional studies revealed that these mutants make up complexes that are involved in the formation of intraluminal vesicles (ILVs) from the limiting membrane of the endosome and the sorting of downregulated cell surface receptors into ILVs (Babst et al., 2002a; Babst et al., 2002b; Katzmann et al., 2001; Katzmann et al., 2003; reviewed in Hanson and Cashikar, 2012). These complexes were named ESCRTs for endosomal sorting complex required for transport (Katzmann et al., 2001). The yeast ESCRT complexes were later found to have well-conserved homologues that perform similar functions in humans and other higher organisms (reviewed in Hanson and Cashikar, 2012).

Through a series of cell biology and genetic experiments in yeast as well as reconstitution experiments in vitro, the individual roles of the ESCRT complexes in cargo sorting and ILV formation were elucidated (for comprehensive reviews see Hanson and Cashikar, 2012; and Hurley and Hanson, 2010). Briefly, ILV formation is initiated by ESCRT-0 through recognition of ubiquitinated cargo and phosphatidylinositol 3-phosphate (PI3P), a lipid enriched on endosomes (Katzmann et al., 2003; Wollert and Hurley, 2010). ESCRT-I and ESCRT-II are recruited to the endosome to begin the deformation of the endosomal membrane and to sort ubiquitinated cargo into these membrane buds (Katzmann et al., 2003; Wollert and Hurley, 2010). ESCRT-II then recruits and activates ESCRT-III (Babst et al., 2002b; Im et al., 2009; Teis et al., 2010; Teo et al., 2004; Wollert and Hurley, 2010), which oligomerizes and pinches off the membrane neck to release the ILV into the lumen of the endosome (Teis et al., 2008; Wollert and Hurley, 2010; Wollert et al., 2009). As a final step, Vps4 hydrolyzes ATP to release the ESCRT complexes from the membrane for additional rounds of ILV formation (Babst et al., 1997; Babst et al., 1998; Wollert and Hurley, 2010; Wollert et al., 2009). The resulting multivesicular bodies (MVBs) can ultimately fuse with the lysosome to degrade the internalized receptors or with the
plasma membrane to release their contents into the extracellular space (Futter et al., 1996; reviewed in Hanson and Cashikar, 2012).

The concept that ESCRT-II may play a non-endosomal role in RNA trafficking is not unprecedented, as ESCRT complexes have been highlighted as having multiple cellular functions. As a group, ESCRT complexes perform roles in cytokinesis (Carlton and Martin-Serrano, 2007; Morita et al., 2007) and viral release (Votteler and Sundquist, 2013) in addition to MVB formation, although ESCRT-II appears to be dispensable for these two non-endosomal functions (Carlton and Martin-Serrano, 2007; Langelier et al., 2006; Morita et al., 2007). Furthermore, the ESCRT complexes (including ESCRT-II) localize to centrosomes and influence centrosome number and morphology as well as spindle formation (Frost et al., 2012; Jin et al., 2005; Morita et al., 2010). Along similar lines, several ESCRT complexes were recently found to be localized to the transition zone of cilia, although their function in cilia remains to be investigated (Diener et al., 2015).

Individually, several ESCRT complexes have been found to have independent, non-endosomal roles. Tsg101 of ESCRT-I plays a role in tumor suppression, in part through the regulation of p53 (Li et al., 2001) and p21 (Lin et al., 2013; Oh et al., 2002). The ESCRT-III component CHMP1 was originally identified as a partner of the polycomb-group protein Polycomblike and was found to influence chromatin structure and gene silencing (Stauffer et al., 2001). In addition to an involvement in bicoiRNA localization, ESCRT-II itself has been found to play at least one other non-endosomal role; in humans, the ESCRT-II components were first identified as interactors of the transcription elongation factor ELL, and, consequently, the names of the human ESCRT-II subunits – EAP20 (for ELL associated protein), EAP30, and EAP45 – reflect this initial discovery (Kamura et al., 2001; Schmidt et al., 1999; Shilatifard, 1998).
The structure and interactions of ESCRT-II

The idea that ESCRT-II might interact with nucleic acids was first revealed when the crystal structures of yeast and human ESCRT-II were solved (Hierro et al., 2004; Im and Hurley, 2008; Teo et al., 2004) (Figure 1.2A). The ESCRT-II subunits are arranged in the shape of the letter “Y”, with Vps22 and Vps36 forming a tight interaction to make up the base of the “Y”, and the two copies of Vps25 making up the more exposed arms of the “Y”. Importantly, these crystal structures revealed that ESCRT-II contains 8 winged-helix (WH) domains, with 2 WH domains on each subunit of ESCRT-II. While the WH domains of each subunit have distinct sequences,
the structures of the WH domains superimpose each other surprisingly well (Hierro et al., 2004; Im and Hurley, 2008; Teo et al., 2004). WH domains are well-characterized nucleic acid binding domains (reviewed in Harami et al., 2013), providing the possibility that ESCRT-II might interact with nucleic acids through these domains.

While ESCRT-II could potentially interact with RNA through any of its subunits, ESCRT-II has a variety of interacting partners that may interfere with such an interaction \textit{in vivo} (Figure 1.2B). During MVB formation, ESCRT-II interacts with ubiquitinated cargo, phospholipids, ESCRT-I, and ESCRT-III. In humans, the GLUE domain of Vps36 binds to ubiquitin (Alam et al., 2006; Hirano et al., 2006; Slagsvold et al., 2005) and, along with the H0 domain of Vps22, binds to phospholipids (Im and Hurley, 2008; Slagsvold et al., 2005; Teo et al., 2006). Vps36 also mediates the interaction with Vps28 of ESCRT-I (Im and Hurley, 2008). Similar interactions occur for yeast ESCRT-II, except that the GLUE domain includes two extra domains in a yeast specific insertion, NZF-C and NZF-N, which bind to ubiquitin (Alam et al., 2004) and ESCRT-I (Gill et al., 2007; Teo et al., 2006), respectively. In both humans and yeast, Vps25 directly interacts with Vps20 of ESCRT-III (Im et al., 2009; Teo et al., 2004; Yorikawa et al., 2005). As mentioned above, ESCRT-II also interacts with RILP (Progida et al., 2006; Wang and Hong, 2006), a protein that interacts with the dynein/dynactin complex (Jordens et al., 2001). Vps22 and Vps36 were suggested to mediate this interaction, but more extensive biochemical data are needed to confirm these interaction sites. Where an interaction with RNA fits into this picture is unclear, although Irion and colleagues suggest that ESCRT-II interacts with \textit{bicoid} RNA through the Vps36 GLUE domain (Irion and St Johnston, 2007).
Links between membranes and RNA regulation

The finding that an endosomal sorting complex is involved in RNA localization hints that membrane trafficking may be linked to this process. Indeed, correlations between membranes and a variety of RNA localization pathways have been observed. For example, in the early vegetal RNA localization pathway in *Xenopus* oocytes, RNAs localize to the Balbiani body, a structure in the oocyte that contains mitochondria, ER, Golgi complexes, and other membranous organelles (reviewed in Kloc et al., 2004). The Balbiani body traps and transports early localizing RNAs, including Xlsirts, Xcat2 and Xwnt11, as it moves towards the vegetal cortex (Chang et al., 2004; Kloc and Etkin, 1995). Later in oocyte development, Vg1 mRNA localizes to a wedge-like structure between the vegetal cortex and the nucleus (Kloc and Etkin, 1995). The ER forms a similar wedge-like structure at these stages, and colocalization of Vg1 mRNA and the ER has been observed prior to enrichment of Vg1 at the vegetal cortex (Deshler et al., 1997; Kloc and Etkin, 1998). While these correlative data suggest an involvement of the ER in Vg1 mRNA localization, additional studies are needed to address whether Vg1 mRNA is tethered to the ER and if the ER is required for Vg1 mRNA localization.

In budding yeast, a subset of bud-localized RNAs comigrate with the ER in a translation-independent manner during their trafficking to the daughter cell during cell division (Fundakowski et al., 2012; Schmid et al., 2006), and while *ASH1* is transcribed after ER transport is complete, if expressed early *ASH1* will comigrate with the ER as well (Schmid et al., 2006). As further evidence of an involvement of the ER in RNA trafficking in yeast, She2p, a member of the RNA transport machinery in yeast, has membrane binding domains (Genz et al., 2013) and cosediments with the ER (Schmid et al., 2006). Furthermore, mutations in proteins that affect membrane trafficking also impair RNA localization in yeast (Trautwein et al., 2004).
Thus, the ER and additional membranous organelles have been implicated in a variety of RNA localization pathways.

**Involvement of endosomes in RNA localization and regulation**

While there are several examples of an involvement of the ER in RNA localization, more recently endosomes have been linked to RNA localization as well. In addition to the involvement of ESCRT-II in *bicoid* localization, links between RNA and endosomes have been observed in the posterior localization of *oskar* mRNA in *Drosophila* oocytes. Flies with mutations in the endosome-associated proteins Rab11 (Dollar et al., 2002; Jankovics et al., 2001) or Rabenosyn-5 (Tanaka and Nakamura, 2008) have defects in *oskar* mRNA localization, although these mutants additionally display a reorganization of the microtubule cytoskeleton which is likely to be the primary cause of *oskar* mislocalization (Dollar et al., 2002; Jankovics et al., 2001; Tanaka and Nakamura, 2008). Additionally, Mon2, an endosome-associated protein, influences anchoring of *oskar* mRNA at the posterior pole by remodeling the cortical actin (Tanaka et al., 2011). Each of these cases suggest that *oskar* mRNA does not directly associate with endosomes to undergo localization, but rather depends indirectly on endosome-associated proteins for proper localization.

A more direct association of RNA with endosomes was recently observed in the filamentous fungus *Ustilago maydis*. RRM4, an RNA-binding protein, influences polarity in the hyphal stage of this organism (Becht et al., 2005), and the RNA-binding properties of RRM4 are essential for this function (Becht et al., 2006). In subsequent studies, it was found that RRM4 associates with endosomes (Baumann et al., 2012), and the mRNA targets of RRM4 traffic with RRM4-positive endosomes (Baumann et al., 2014). Finally, two groups demonstrated that
ribosomes colocalize and travel with the RRM4 positive endosomes (Baumann et al., 2014; Higuchi et al., 2014), as do the protein products of RRM4-associated mRNAs (Baumann et al., 2014), suggesting that local translation might occur on these endosomes. Whether or not a similar mode of transport occurs in higher organisms is unclear. However, the authors of these studies point out that *mbp* mRNA trafficking in zebrafish neurons requires Kif1B (Jansen et al., 2014; Lyons et al., 2009), a molecular motor that is also involved in the transport of synaptic vesicles (Zhao et al., 2001). It has been suggested that retroviruses might utilize endosomal trafficking to transport their viral RNA to assembly sites (Basyuk et al., 2003; Lehmann et al., 2009; Molle et al., 2009), but evidence against this possibility has also been presented (Chen et al., 2014). These studies suggest that the role of endosomes in RNA transport warrants further investigation.

In higher organisms, a more clear example of RNAs associating with endosomes has been revealed through the study of exosomes. Exosomes are extracellular vesicles that are derived from intraluminal vesicles and are released from the cell when an MVB fuses with the plasma membrane (Harding et al., 1983; Johnstone et al., 1987; Pan et al., 1985; reviewed in Colombo et al., 2014). Both mRNA and miRNAs were first detected in exosomes derived from human and mouse mast cells (MC/9, BMMC, and HMC-1 cells) (Valadi et al., 2007). The RNA population within these exosomes differed from the population of total RNA in the cells that the exosomes were derived from, suggesting that there is a mechanism to selectively sort RNAs into exosomes. Since this initial discovery, RNA has been detected in exosomes, as well as additional extracellular vesicles, from a wide variety of cell types (reviewed in Raposo and Stoorvogel, 2013). While the biological significance of exosomal RNA is still unclear, it has been suggested
that RNAs are sorted into exosomes in order to transfer to other cells as a means of cell-to-cell communication (Valadi et al., 2007).

In addition to an interaction with exosomal RNAs, endosomes associate with RNAs during miRNA-mediated silencing. In two complementary studies, microRNA-mediated silencing was found to occur on endosomes and to depend on MVB formation (Gibbings et al., 2009; Lee et al., 2009). In one study, a genetic screen in *Drosophila* for factors that enhance siRNA-mediated silencing implicated a role for endosomal trafficking in this process. Further investigation revealed that knocking down ESCRT components impaired miRNA-mediated silencing, that GW-bodies associate with and are influenced by MVBs, and that MVBs influence miRISC loading (Lee et al., 2009). In the other study, the presence of miRNAs in exosomes (Valadi et al., 2007) led the authors to explore an involvement of MVBs in miRISC activity. They found that GW182 is also enriched in exosomes, and that GW182, Ago2, miRNAs, and mRNA targets of miRNAs cofractionate and colocalize with endosomal markers. Similar to the other study, they found that knockdown of ESCRT components reduced miRNA activity (Gibbings et al., 2009).

Thus, RNA associates with endosomes for a variety of purposes. RNAs interact with endosomes to undergo long distance transport in the cell, to be selectively sorted into intraluminal vesicles and released as exosomes, or to interact with the RISC machinery. In each of these cases, a selective interaction between a subset of RNAs and the endosome is achieved. Therefore, mechanisms must exist in the cell to recognize RNAs that are destined for the endosome and to transport RNAs to this destination.
Summary and goal of this thesis

RNA localization to distinct regions of the cell, especially to particular organelles, is an important mechanism to compartmentalize cellular components for a variety of biological purposes. Understanding the factors involved in this selective sorting of RNAs is essential to understanding how cellular organization occurs. ESCRT-II is a recently discovered trans-acting factor involved in RNA localization, yet the possibility that ESCRT-II is involved in RNA regulatory events outside of Drosophila and bicoid localization has not been explored. Due to the recent implications of endosomes in RNA regulatory events, the fact that a core member of the ESCRT machinery is an RNA-binding factor is particularly intriguing.

The goal of this thesis is to better understand ESCRT-II as an RNA-binding complex. To achieve this goal, I investigated the RNA regulatory roles of ESCRT-II in Xenopus laevis eggs and characterized the molecular mechanism of the ESCRT-II/RNA interaction. Xenopus eggs offer an advantageous system to study ESCRT-II because this is a system where numerous examples of localized RNAs exist, large quantities of undiluted cytoplasmic extract can be generated to facilitate studies of RNA/protein interactions, and the concentration of ESCRT-II is particularly high. A better understanding of the targets, molecular mechanism, and purpose of ESCRT-II/RNA interactions will have important implications for both RNA and membrane trafficking pathways and will add to the accumulating evidence that these two pathways are interconnected.
Chapter 2: ESCRT-II interacts with RNAs in *Xenopus laevis* eggs by recognizing purine-rich sequences through the subunit Vps25

Attributions

All experiments in this chapter were performed by Amy Emerman. Mike Blower performed all bioinformatics analyses. Josh Plant provided preliminary data that ESCRT-II interacts with RNAs in *Xenopus* eggs and generated the *Xenopus* ESCRT-II construct and the first batch of the ESCRT-II polyclonal antibody. All members of the Blower lab offered critical advice throughout this project.

Introduction

A major mechanism used by the cell to regulate gene expression is through the subcellular localization of mRNAs. mRNA localization has been recognized as a widespread phenomenon, occurring in organisms ranging from bacteria to humans, and in a wide range of cell types (reviewed in Cody et al., 2013). RNA localization has been particularly highlighted in polarized cells, such as germ cells, migrating cells, and neurons, but many RNAs show subcellular localization patterns in additional cell types by selectively partitioning onto organelles and subcellular structures (reviewed in Cody et al., 2013; Weis et al., 2013). For example, subsets of RNAs are enriched on the ER, the mitochondria, and the cytoskeleton (reviewed in Cody et al., 2013; Weis et al., 2013). An important focus in the field is to understand how particular RNAs are selected to be transported to subcellular locations. One of the best-studied mechanisms to achieve these localization patterns is through recognition of *cis*-acting sequences on the mRNA by *trans*-acting factors (reviewed in Martin and Ephrussi, 2009).
In a screen for novel factors that are involved in RNA localization, the ESCRT-II complex was identified to be necessary for proper localization of *bicoid* RNA (Irion and St Johnston, 2007). ESCRT-II is a four subunit complex consisting of two copies of Vps25 and one copy each of Vps22 and Vps36 (Hierro et al., 2004; Im and Hurley, 2008; Teo et al., 2004). ESCRT-II is one of four complexes (ESCRTs 0-III) that are involved in maturing endosomes into multivesicular bodies (MVBs) by sorting downregulated receptors into intraluminal vesicles within the endosome (reviewed in Hurley, 2008). Mutations in additional ESCRT complexes have no effect on *bicoid* mRNA localization, suggesting that the role of ESCRT-II in RNA localization is independent of its role in MVB formation (Irion and St Johnston, 2007). ESCRT-II was previously uncharacterized as an RNA-binding complex, however crystal structures revealed that ESCRT-II contains winged-helix domains (Hierro et al., 2004; Im and Hurley, 2008; Teo et al., 2004), which are well-characterized nucleic acid binding domains (reviewed in Harami et al., 2013). In addition, ESCRT-II is known to play a moonlighting role in the nucleus where it interacts with the transcription elongation factor, ELL (Kamura et al., 2001; Schmidt et al., 1999; Shilatifard, 1998), but its role in this process is unclear.

Recent studies have implicated endosomes in a variety of RNA regulatory events, and the finding that ESCRT-II influences RNA localization in *Drosophila* raises the intriguing possibility that ESCRT-II could provide a link between endosomes and RNA regulation. For example, miRNA-mediated silencing has been found to occur on endosomes and to require components of the ESCRT machinery (Gibbings et al., 2009; Lee et al., 2009). In addition, exosomes, which are derived from intraluminal vesicles and are released when MVBs fuse with the plasma membrane, have been found to contain selective populations of mRNAs and miRNAs (Valadi et al., 2007; reviewed in Raposo and Stoorvogel, 2013). Finally, endosomes have been
implicated in RNA trafficking pathways. For example, in the filamentous fungus *Ustilago maydis*, mRNAs are locally translated on endosomes as they transport through a polarized hyphae (Baumann et al., 2014).

While the implications of a protein having dual roles in endosomal trafficking and RNA regulation are intriguing, to date, *bicoid* is the only known direct target of ESCRT-II. Many *trans*-acting factors are conserved for their roles in RNA localization pathways (reviewed in St Johnston, 2005), raising the possibility that ESCRT-II may be involved in RNA localization pathways in additional organisms. In this study, we sought to further characterize the RNA-binding properties of ESCRT-II and to determine if the direct role of ESCRT-II in RNA regulation is conserved across species.

Here we examined the RNA-binding properties of ESCRT-II in *Xenopus laevis* eggs, a well-studied system in which many RNAs show particular subcellular localization patterns (reviewed in King et al., 2005). Large amounts of undiluted cytoplasmic egg extract can be prepared from *Xenopus* eggs, allowing ample material for the study of RNA/protein interactions. Furthermore, we found that the concentration of ESCRT-II is particularly high in these eggs, which avoided the need to introduce tagged and overexpressed constructs into the system and facilitated the identification of direct targets of endogenous ESCRT-II.

We used a combination of RNA immunoprecipitation (RIP)-Seq and UV-crosslinking immunoprecipitations followed by high-throughput sequencing (CLIP-seq) (Ule et al., 2005; Ule et al., 2003) to identify novel targets of ESCRT-II. CLIP-seq offers the advantage of stringently identifying direct targets of a protein as well as the footprint of the protein on an RNA. CLIP-seq has been successfully used to identify direct targets of a variety of RNA-binding proteins (reviewed in Milek et al., 2012), and novel molecular details of factors involved in RNA
localization have been revealed through CLIP-seq studies (Ascano et al., 2012; Darnell et al., 2011; Racca et al., 2010). However, limiting quantities of RNA recovered from CLIP-seq experiments can result in low coverage of the transcriptome, resulting in many false negatives in the dataset. RIP-seq can be used to more extensively analyze genome-wide associations of a protein, although RIP-seq identifies both direct and indirect targets and can include more noise due to nonspecific interactions (reviewed in Darnell, 2010). Therefore, we combined data from both approaches to reveal direct targets of ESCRT-II, the RNA motif that ESCRT-II recognizes, the subunit of ESCRT-II that is responsible for binding to RNA, as well as an extensive coverage of the genome-wide associations of ESCRT-II. Using these approaches, we found that ESCRT-II interacts with hundreds of RNAs in *Xenopus* eggs by recognizing a polypurine sequence through the subunit Vps25.

**Results**

**ESCRT-II associates with mRNAs in *Xenopus* egg extracts**

To investigate ESCRT-II/RNA interactions in *Xenopus* eggs, we first determined if the endogenous concentration of ESCRT-II in eggs is high enough to perform such experiments. We generated an undiluted meiotic cytoplasmic egg extract and performed a Western blot for ESCRT-II in egg extract next to a range of recombinant ESCRT-II concentrations. We also compared the concentration of ESCRT-II in egg extract to its concentration in undiluted *Xenopus* oocyte lysates. We found that the concentration of ESCRT-II is approximately 200nM in *Xenopus* egg extract (Figure 2.1). In contrast, the ESCRT-II concentration in yeast was estimated to be approximately 79nM (Wollert and Hurley, 2010). The high concentration of ESCRT-II in *Xenopus* egg extracts makes *Xenopus* eggs an ideal system to investigate ESCRT-II/RNA interactions.
Figure 2.1: Concentration of ESCRT-II in Xenopus egg extract. Cytoplasmic lysates were prepared from X. laevis stage I-III oocytes, stage IV-VI oocytes, or eggs as described in the materials and methods (Chapter 5). Oocytes were staged according to size and were separated using a 450 µM mesh screen. Lysates from each stage were run on SDS-PAGE next to equivalent volumes of a range of concentrations of recombinant X. laevis ESCRT-II. Western blot analysis was performed using a polyclonal ESCRT-II antibody that was raised against the entire ESCRT-II complex, and detection of the ESCRT-II subunit Vps25 is shown. Equal loading of the oocyte/egg stages were confirmed by Ponceau S staining.

interactions. While ESCRT complexes have been understudied in Xenopus eggs, it is possible that the concentration is high due to the involvement of MVBs in yolk platelet production (Wall and Patel, 1987). Interestingly, the concentration of ESCRT-II increases throughout oocyte maturation, though the purpose of the increase from oocytes to eggs is unclear (Figure 2.1).

To determine if ESCRT-II associates with RNAs in Xenopus laevis eggs, we performed an RNA immunoprecipitation (RIP) for ESCRT-II from egg extract. ESCRT-II immunoprecipitations are enriched for high molecular weight RNAs compared to total RNA, which is indicative of an enrichment of mRNA in ESCRT-II immunoprecipitations (Figure 2.2A). In contrast, a mock immunoprecipitation performed with nonspecific rabbit IgG co-immunoprecipitated with very little RNA. We submitted the ESCRT-II-associated RNAs for
Figure 2.2: ESCRT-II interacts with mRNAs in Xenopus egg extract.
Endogenous ESCRT-II was immunoprecipitated from Xenopus egg extract and the associated transcripts were isolated. A) Agarose gel of RNA isolated from total egg extract compared to mock or ESCRT-II immunoprecipitations. Equal mass was loaded between the total RNA and ESCRT-II RNA samples, and equal volume was loaded between the ESCRT-II and Mock RNA samples. B and C) High-throughput sequencing results of RNA libraries prepared from total egg extract or ESCRT-II immunoprecipitations. B) Scatterplot of normalized transcript abundance (RPKM) in an ESCRT-II immunoprecipitation (IP) compared to total egg RNA. Red circles indicate transcripts that are highly enriched in ESCRT-II immunoprecipitations relative to their abundance in total egg extract (>2 standard deviations above the mean enrichment value). Blue line depicts equal RPKM in both samples. C) Correlation of ESCRT-II enrichment (RPKM in ESCRT-II IP/Total) across two biological replicates. The Pearson correlation coefficient is 0.92. D) qPCR validation of ESCRT-II enrichment (abundance in ESCRT-II immunoprecipitations/abundance in total RNA) of transcripts predicted by RIP-seq to be ESCRT-II-associated. Negative control RNAs are 28S ribosomal RNA (rRNA) or transcripts predicted by RIP-seq to be underenriched in ESCRT-II immunoprecipitations. n= 3 independent experiments. Error bars represent SEM.
Figure 2.2 (Continued)
high-throughput sequencing and found that 556 mRNAs are significantly enriched in an ESCRT-II immunoprecipitation (2 standard deviations above a mean enrichment value of 1.59) (Figure 2.2B). Two biological replicates of ESCRT-II RNA immunoprecipitations show a strong correlation in RNA enrichment in the immunoprecipitations, demonstrating that these data are highly reproducible ($r=0.92$) (Figure 2.2C). We validated the high-throughput sequencing results by qPCR and found that ESCRT-II reproducibly interacts with top hits from the RIP-Seq library, but not with RNAs that are underrepresented in the library (Figure 2.2D).

To determine if ESCRT-II interacts with a specific class of RNAs, we submitted the list of ESCRT-II-associated RNAs for gene ontology analysis (Huang da et al., 2009a, b), which revealed that ESCRT-II-associated RNAs encode proteins involved with cell adhesion, membranes, the cytoskeleton/centrosome, and cell motility (Table 2.1).

**Table 2.1: Gene ontology analysis of the ESCRT-II RIP-seq library**

Summary of the top four annotation clusters in overrepresented and underrepresented transcripts in the ESCRT-II RIP-seq library, identified by NCBI DAVID (Huang da et al., 2009a, b).

<table>
<thead>
<tr>
<th>Annotation cluster</th>
<th>Enrichment score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overrepresented clusters:</strong></td>
<td></td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>6.85</td>
</tr>
<tr>
<td>Membranes</td>
<td>3.86</td>
</tr>
<tr>
<td>Cytoskeleton/Centrosome</td>
<td>3.63</td>
</tr>
<tr>
<td>Cell Motility</td>
<td>3.18</td>
</tr>
<tr>
<td><strong>Underrepresented clusters:</strong></td>
<td></td>
</tr>
<tr>
<td>Ribosomal/cytosolic proteins</td>
<td>25.15</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>3.51</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>2.37</td>
</tr>
<tr>
<td>rRNA/ncRNA processing</td>
<td>2.24</td>
</tr>
</tbody>
</table>
Figure 2.3: ESCRT-II is primarily cytoplasmic in *Xenopus* egg extract.

Cytosolic (Cyt) and light membrane (Mem) fractions of egg extract were prepared as previously described (Schwarz and Blower, 2014), and a Western blot was performed for the ESCRT-II subunit Vps25. Markers for the endoplasmic reticulum (Trapα), late endosomes (Rab7), or cytosol (α-tubulin) are shown as validation for the fractionation.

Given that one of the major classes of RNAs that interact with ESCRT-II encode proteins involved in membrane biology, we asked if ESCRT-II associates with membranes in egg extract. To address this question, we performed a Western blot for ESCRT-II on membrane or cytosolic fractions of egg extract. The majority of ESCRT-II is cytoplasmic in egg extracts (Figure 2.3), similar to findings described in yeast and human tissue culture cells that ESCRT-II is primarily cytoplasmic and only transiently interacts with membranes (Babst et al., 2002b; Langelier et al., 2006).

Taken together, these data demonstrate that ESCRT-II interacts with hundreds of RNAs in *Xenopus* egg extract, and while ESCRT-II interacts with several RNAs that are likely to be
localized to subcellular structures (such as transcripts associated with membrane biology), the interaction between ESCRT-II and its RNA targets is likely to occur primarily in the cytosol.

**ESCRT-II directly interacts with RNA through Vps25**

We next asked if ESCRT-II interacts directly with its RNA targets. In addition, we asked which subunit of ESCRT-II – Vps22, Vps25, or Vps36 – is responsible for RNA binding in *Xenopus* eggs. To address both questions simultaneously, we took a UV-crosslinking approach in *Xenopus* egg extract similar to CLIP (Ule et al., 2005; Ule et al., 2003). Our polyclonal ESCRT-II antibody was raised against the entire ESCRT-II complex, but primarily recognizes Vps25 and more weakly recognizes Vps36 by Western blot. However, immunoprecipitation for any ESCRT-II subunit co-immunoprecipitates the entire complex; therefore, any subunit that interacts with RNA will be detectable by this assay. This approach revealed a radiolabeled band consistent with the molecular weight of Vps25 crosslinked to a short fragment of RNA (approximately 20kDa) (Figure 2.4A). This band was absent in the mock immunoprecipitation as well as a no-crosslinking control. Additionally, this band was absent in a control lacking T4 RNA ligase and shifted in a low-RNase control, suggesting that the protein is radiolabeled through an interaction with RNA (not shown). In addition to the Vps25 band, we found a lighter band that migrates higher than the expected molecular weight of Vps22 crosslinked to an RNA fragment, and we never observed a convincing band near the molecular weight of Vps36. Together these results suggest that Vps25 is the RNA-binding subunit of ESCRT-II.

While we found a radiolabeled band that migrates at the molecular of Vps25, we sought to confirm that this band is in fact Vps25 and not a co-immunoprecipitating protein of the same size. To address this question, we repeated the UV-crosslinking experiment under denaturing
Figure 2.4: The ESCRT-II subunit Vps25 binds directly to RNA.

A) *Xenopus* egg extract was UV crosslinked, then following a high RNaseA treatment, ESCRT-II or mock immunoprecipitations were performed. The RNA fragments were 3’ end labeled with T4 RNA ligase and [32P]pCp as described in the materials and methods (Chapter 5), then the samples were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. The expected migrations of the ESCRT-II subunits are indicated to the left of the gel. A radioactive band consistent with the molecular weight of Vps25 (denoted by the red asterisk) is observed, while no bands at the molecular weights of Vps22 or Vps36 are apparent. T4 RNA ligase forms a covalent intermediate with pCp and appears in every lane. The bands above and below the Vps25 band (black asterisks) are considered to be contaminating bands because they appeared in the IgG control in some replicates of this experiment. B) Western blot of ESCRT-II immunoprecipitations (IPs) under native (-) or denaturing (+) conditions, demonstrating that Vps36 no longer co-immunoprecipitates with Vps25 under denaturing conditions. Vps22 is not detectable by Western blot with our ESCRT-II antibody. H.C.= heavy chain. C) Autoradiograph of a CLIP experiment from *Xenopus* egg extract performed as described in (A), except under denaturing immunoprecipitation conditions, confirming that Vps25 (red asterisk) binds directly to RNA. The same polyclonal ESCRT-II antibody was used for (A-C), but under denaturing conditions this antibody only recognizes Vps25 by immunoprecipitation.
Figure 2.4 (Continued)
immunoprecipitation conditions. This approach involved disrupting protein-protein interactions with detergent and heat prior to performing the immunoprecipitations. When we immunoprecipitate Vps25 under these conditions, we no longer co-immunoprecipitate Vps36, demonstrating that these conditions are sufficient to disrupt interactions among the ESCRT-II subunits (Figure 2.4B). It is therefore unlikely that unknown interactors of ESCRT-II co-immunoprecipitate with Vps25 under these denaturing conditions. We found that the Vps25 band remains when CLIP is performed under denaturing immunoprecipitation conditions, providing further evidence that Vps25 mediates RNA binding by ESCRT-II in Xenopus eggs (Figure 2.4C).

ESCRT-II directly binds to hundreds of RNAs

To identify the direct RNA targets of ESCRT-II, we performed CLIP-seq on the UV-crosslinked material under denaturing conditions (Figure 2.5A). Using the Illumina MiSeq platform, we obtained approximately 10 million reads, with 237,969 unique sequences and 44,535 unique sequences that were greater than 10 base pairs in length. Of these unique sequences, 95.3% was ribosomal RNA. After removal of empty adapters, short inserts, ribosomal RNA, and homopolymers, we had 11,167 unique reads.

We aligned the unique reads to a custom transcriptome derived from the Xenopus laevis 7.0 genome and our own sequencing data (Schweidenback et al., 2015). The unique reads aligned to 1640 transcripts (Figure 2.5B), of which 196 transcripts had more than one aligning read. In addition, 114 transcripts had multiple CLIP-tags (unique aligned reads) that overlapped at a particular region of the transcript (pileups), which is suggestive of a footprint of ESCRT-II on the mRNA (see Figure 2.5C for examples).
Figure 2.5: ESCRT-II CLIP-seq.

A) Left (membrane): ESCRT-II or mock UV-CLIP samples were prepared using low-RNase and denaturing immunoprecipitation conditions. RNAs were 5’ end labeled with PNK and [γ-32P]-ATP. Following SDS-PAGE and transfer to nitrocellulose, the membrane was analyzed by autoradiography. RNA was isolated from the indicated regions of the nitrocellulose membrane (red, numbered boxes) as described in the materials and methods (Chapter 5). Right (gel): Isolated RNA from the indicated regions of the nitrocellulose membrane was run on a 12% urea acrylamide gel and visualized by autoradiography. The expected nucleotide lengths of the isolated RNAs for each region of the nitrocellulose membrane are indicated, and is approximated based on the difference between the molecular weight of the membrane region and the molecular weight of Vps25 alone, assuming 3 nucleotides adds approximately 1 kDa. The actual CLIP-seq was performed using region 2 of the nitrocellulose membrane from a non-radioactively labeled denaturing UV-crosslinking immunoprecipitation.

B) Histogram of the number of unique reads per transcript in the ESCRT-II CLIP-seq library. Due to the presence of paralogues in the Xenopus laevis transcriptome, unique reads were allowed to align to up to two transcripts. Weighted counting was performed such that if a read aligned twice, each of the two transcripts would display 0.5 aligned reads. The values in this histogram reflect the weighted read counts.

C) Alignment of CLIP-tags to the transcripts XLOC_000692, XLOC_045962, and XLOC_045037, demonstrating pile-ups of CLIP-tags on the transcripts. 114 transcripts in the CLIP-seq library contained overlapping alignments.
Figure 2.5 (Continued)

A

Expected nt length:
1) 0-27
2) 27-42
3) 42-69
4) 69-117

B

# of unique reads per transcript

C

Coverage [0-10]

ESCRT-II

CLIP reads

XLOC_000692

XLOC_045862

XLOC_046037

ESCRT-II

CLIP reads
We next asked if the ESCRT-II CLIP-tags come from mRNAs that are enriched in an ESCRT-II immunoprecipitation. Transcripts that have two or more CLIP-tags are significantly more enriched in an ESCRT-II immunoprecipitation ($P$-value $< 0.0014$) compared to the full RIP-seq dataset (Figure 2.6A). Similarly, we observe that transcripts containing ESCRT-II CLIP-tags are enriched in ESCRT-II immunoprecipitations when assayed by qPCR (Figure 2.6B).

**ESCRT-II interacts with RNAs that contain a GA-rich motif**

Based on our RIP-seq data, it appears that ESCRT-II has a selective interaction with its RNA targets. This selective interaction could be achieved through the recognition of a sequence or structural element on the RNA target, through additional *trans*-acting factors associated with the transcripts, or could be influenced by the local population of transcripts in the vicinity of ESCRT-II *in vivo*. To determine if ESCRT-II has a sequence preference for RNA, we performed a motif analysis of the CLIP-seq library. The top motif was a stretch of G’s and A’s, and appeared in 113/1640 of the CLIP-seq transcripts (Figure 2.7A). The second hit was a C-rich sequence that appeared in 96 transcripts, and a third motif occurred in 104 transcripts (Figure 2.7A).

The identification of sequence motifs that are enriched in our ESCRT-II CLIP-seq library raises the possibility that the selective interaction between ESCRT-II and its RNA targets is dependent on this sequence. We therefore asked if the GA-rich motif is enriched in the ESCRT-II RIP-seq library. When we compared the distribution of ESCRT-II enrichment of our entire RIP-seq library to a subset of transcripts in the RIP-seq library that have a 30 nucleotide stretch of at least 90% purines, we found that transcripts with this motif tend to be more enriched in an
Figure 2.6: Transcripts with ESCRT-II CLIP-tags are enriched in ESCRT-II immunoprecipitations.

A) Cumulative distribution plot demonstrating that RIP-seq transcripts with $\geq 2$ CLIP-tags are overall more enriched in the ESCRT-II RIP-seq library compared to all ESCRT-II RIP-seq transcripts ($P$-value < 0.0014 using a two-sided unpaired t-test) B) qPCR validation of ESCRT-II enrichment (abundance in ESCRT-II immunoprecipitation/abundance in total RNA) of transcripts containing CLIP-tags compared to a negative control RNA (28S rRNA). Two biological replicates are shown.
Figure 2.7: ESCRT-II recognizes a GA-rich motif

A) Overlapping ESCRT-II CLIP-reads were collapsed into single CLIP-tags and extended with 25 nts of flanking sequences on the aligned transcript. The resulting sequences were used as input for MEME motif analysis (Bailey et al., 2009). The top three motifs in the ESCRT-II CLIP-seq library are shown. B) Cumulative distribution plot demonstrating that RIP-seq transcripts containing a GA-rich motif are generally more enriched in the ESCRT-II RIP-seq library compared to all ESCRT-II RIP-seq transcripts (P-value < 1 x 10^{-12} using a two-side unpaired t-test).
Table 2.2: Transcripts containing GA-rich or C-rich motifs are more enriched in ESCRT-II immunoprecipitations compared to the total RIP-seq library. Means of the distributions of RIP-seq transcripts containing 30 nucleotide stretches of the indicated nucleotides or dinucleotides (see methods regarding allowance of mismatches) compared to the mean of the distribution of all ESCRT-II RIP-seq transcripts. P-values were determined using a two-sided, unpaired t-test comparing the distributions of the indicated RIP-seq transcripts to the distribution of all ESCRT-II RIP-seq transcripts.

<table>
<thead>
<tr>
<th>Transcripts</th>
<th>Mean enrichment value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All RIP-seq transcripts</td>
<td>1.59</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>1.97</td>
<td>9.19 x 10^-13</td>
</tr>
<tr>
<td>AC</td>
<td>1.78</td>
<td>0.06</td>
</tr>
<tr>
<td>AU</td>
<td>1.61</td>
<td>0.55</td>
</tr>
<tr>
<td>CU</td>
<td>1.6</td>
<td>0.88</td>
</tr>
<tr>
<td>GU</td>
<td>1.75</td>
<td>0.02</td>
</tr>
<tr>
<td>CG</td>
<td>1.67</td>
<td>0.2</td>
</tr>
<tr>
<td>A</td>
<td>1.65</td>
<td>0.24</td>
</tr>
<tr>
<td>U</td>
<td>1.68</td>
<td>0.21</td>
</tr>
<tr>
<td>G</td>
<td>1.6</td>
<td>0.88</td>
</tr>
<tr>
<td>C</td>
<td>1.83</td>
<td>7.76 x 10^-5</td>
</tr>
</tbody>
</table>

ESCRT-II immunoprecipitation compared to the total RIP-seq library (Figure 2.7B) (P-value < 1 x 10^{-12}). This was not true if we performed the same analysis with any other dinucleotide or single nucleotide motif, with the exception of stretches of cytosines, our second motif identified in the CLIP-seq library (Table 2.2).

ESCRT-II nonspecifically interacts with RNA in vitro through the membrane binding domain of Vps22

Our results indicate that ESCRT-II is capable of interacting directly with RNA through the subunit Vps25 and that the selective interaction between ESCRT-II and its RNA targets is determined in part by recognition of a purine-rich motif. We next asked if we could observe a
selective binding of RNA by ESCRT-II using purified components (Figure 2.8A). We first
determined if recombinant ESCRT-II interacts selectively with purified RNAs through Vps25 \textit{in vitro}. To address this question, we set up binding reactions between recombinant ESCRT-II and
purified RNA from \textit{Xenopus} egg extracts. We then took a UV-crosslinking approach similar to
the CLIP protocol, except modified for \textit{in vitro} binding reactions, to identify the RNA-binding
subunit of recombinant ESCRT-II. We found that unlike in egg extract, ESCRT-II bound to total
egg RNA \textit{in vitro} primarily through Vps22, with some detectable binding through Vps36 and
Vps25 as well (Figure 2.8B). Furthermore, we found that if we performed the same experiment
with a panel of individual \textit{in vitro} transcribed RNAs that are underrepresented in an ESCRT-II
immunoprecipitation, we observed a similar binding pattern for all of the RNAs through Vps22,
suggesting that this interaction is nonspecific (Figure 2.8C).

Vps22 and Vps36 both contain membrane binding domains; Vps22 binds to membranes
through its H0 domain (Im and Hurley, 2008) and Vps36 binds to membranes through its GLUE
domain (Im and Hurley, 2008; Slagsvold et al., 2005; Teo et al., 2006). These positively charged
domains bind to negatively charged phospholipids in cells, and we reasoned that \textit{in vitro}, these
domains may be inappropriately exposed and could bind nonspecifically to the negatively
charged RNA backbone. Folch fraction liposomes have previously been used to investigate
interactions between ESCRT-II and membranes \textit{in vitro} (Teo et al., 2006; Teo et al., 2004). In
support of the hypothesis that RNA binding by Vps22 and Vps36 \textit{in vitro} occurs through their
membrane binding domains, we found that when increasing concentrations of Folch fraction
liposomes are included in the \textit{in vitro} UV-crosslinking assay, binding to total egg RNA through
Vps22 and Vps36 is reduced (Figure 2.8D).
Figure 2.8: ESCRT-II binds to a GA-rich CLIP-tag in vitro through Vps25 and nonspecifically to RNAs through Vps22.

A) Coomassie gel of recombinant proteins used in the in vitro binding assays. Xen= Xenopus laevis, Hu = Human. ΔMBD= lacks the membrane binding domains of human ESCRT-II.

B-D) In vitro binding reactions between recombinant Xenopus ESCRT-II and A) total egg RNA, C) individual in vitro transcribed RNAs, or D) total egg RNA with liposomes included in the binding reactions at the indicated concentrations were UV crosslinked, then following treatment with RNaseA to remove unbound RNAs, ESCRT-II was immunoprecipitated, the remaining RNA was 5’ end labeled, then the samples were analyzed by SDS-PAGE and autoradiography. PNK and [γ-32P]-ATP (used to 5’ end label the RNAs) form a covalent intermediate that appears at the molecular weight of PNK.

E) Autoradiograph of UV-crosslinked in vitro binding reactions as in (B-D), except performed with in vitro transcribed ctr9 CLIP-tag or negative control (ctr9 with the adenosines mutated) RNAs that were body labeled with [α-32P]-GTP. Recombinant HuESCRT-II (FL) or HuESCRT-IIΔMBD (ΔMBD) was used.

A-E) The expected migrations of the ESCRT-II subunits are indicated.
ESCRT-II binds to GA-rich sequences in vitro through Vps25

Our ESCRT-II/RNA binding data in egg extract suggest that Vps25 has specificity for a GA-rich sequence. To address if ESCRT-II binds to this sequence motif in vitro through Vps25, we performed the in vitro UV-crosslinking experiment with full-length human ESCRT-II or a deletion mutant of human ESCRT-II that lacks the membrane binding domains of Vps22 and Vps36 (ESCRT-II ΔMBD) (Im and Hurley, 2008) (Figure 2.8A). As RNA probes for this assay, we chose an ESCRT-II CLIP-tag that contains a long stretch of purines, ctr9, and compared ESCRT-II binding of this transcript to a mutated version of ctr9 that lacks the majority of the adenosines in the GA-rich motif. Full-length ESCRT-II bound to ctr9 through each of its subunits, and Vps22 bound to the negative control RNA to a similar degree (Figure 2.8E). In contrast, ESCRT-II ΔMBD crosslinked only to ctr9 in vitro, and this binding occurred only through Vps25, mimicking the mechanism of binding that was observed in egg extract (Figure 2.8E).

To further investigate the interaction between Vps25 and GA-rich sequences in vitro, we performed an RNA electrophoretic mobility shift assay (RNA EMSA) with the ctr9 CLIP-tag and compared it to a negative control sequence. We performed these experiments with ESCRT-II ΔMBD to avoid the contribution of nonspecific binding (Figure 2.9A). We found that ESCRT-II binds to the ctr9 CLIP-tag with an apparent Kd of approximately 132nM, but that ESCRT-II fails to bind to the negative control RNA (Figure 2.9B). Furthermore, when we fit a curve to the binding data and allowed the hill coefficient to float, we observed a hill coefficient greater than 1. This result suggests that there may be multiple cooperative binding sites for ESCRT-II on the ctr9 CLIP-tag. As we observed in the in vitro UV-crosslinking experiment, we found that if we
Figure 2.9: RNA EMSA of ESCRT-II with the ctr9 CLIP-tag
A) RNA EMSA with ESCRT-II ΔMBD and the ctr9 CLIP-tag or a negative control sequence of the same length (from a region distant from the CLIP-tag on the smu1 transcript). The ESCRT-II ΔMBD concentrations indicated by the white wedge are two-fold dilutions between 3.2 µM - 50 nM. The sequences of the CLIP-tags (plus flanking regions on the transcript to total 40 nucleotides) are indicated.
B) Quantification of the ctr9 EMSA in A, displayed as the fraction of RNA bound versus the concentration of ESCRT-II ΔMBD. A curve fit using the hill equation allowing the hill coefficient to float is shown.
randomly mutate adenosines of the ctr9 CLIP-tag to other nucleotides, binding by ESCRT-II is lost. In contrast, ESCRT-II binds to a version of the ctr9 CLIP-tag with the guanosines mutated with a similar affinity as wild-type ctr9 (Figure 2.10), suggesting that the adenosines of the motif have a greater contribution to ESCRT-II binding. We repeated the RNA EMSA for a panel of CLIP-tags that contained the GA-rich motif, and found that ESCRT-II binds to each of these CLIP-tags in vitro, although the apparent affinity of ESCRT-II for each of these sequences varies with the length and homogeneity of the sequence (Figure 2.11).

**ESCRT-II primarily interacts with the coding region of transcripts**

To determine if ESCRT-II interacts with a particular region of a transcript, we analyzed the locations of ESCRT-II CLIP-tags. The majority of the ESCRT-II CLIP-tags are located within the coding region of transcripts with a bias towards the start codon (Figure 2.12). GA-rich and C-rich motifs also tend to reside in coding regions, suggesting that the biased location of ESCRT-II CLIP-tags arises from ESCRT-II’s preference for these sequences. In contrast, AU- and GU-rich motifs tend to reside in 3’ UTRs. *Trans*-acting factors typically associate with the 3’ UTR of transcripts, largely because this region is free of ribosomal trafficking. However, several *cis*-acting sequences involved in RNA localization pathways have been identified in coding regions, including those identified for bud-localized RNAs in yeast (reviewed in Jambhekar and Derisi, 2007). Translating ribosomes would likely remove ESCRT-II from the transcript; thus, our data suggest that ESCRT-II may interact with mRNAs that are translationally repressed, and future experiments will be aimed at addressing this concept.
Figure 2.10: RNA EMSA of ESCRT-II with the ctr9 CLIP-tag with guanosines or adenosines mutated

RNA EMSA with ESCRT-II ΔMBD and the ctr9 CLIP-tag or the ctr9 CLIP-tag with the majority of the A) guanosines or B) adenosines mutated to other nucleotides. ESCRT-II ΔMBD concentrations indicated by the white wedge in A are two-fold dilutions between 375nM-6nM and in B are two-fold dilutions between 3.2µM-50nM. C) Mutations in the ctr9 CLIP-tag (in red) for the RNA probes used in A and B.

A

[ESCRT-II] ○ 6nM 3.75nM
RNA: ctr9

RNA: ctr9 with Gs mutated

B

[ESCRT-II] ○ 50nM 3.20µM
RNA: ctr9

RNA: ctr9 with As mutated

C

Ctr9: GAAGGAGGAGAGAAGAGAAAAAGAAGAGGAGAAGAGGC
Ctr9 with Gs mutated: CAAGUACUCUACUAAACAGAAAAACAAUACUACUAAAGAGCC
Ctr9 with As mutated: UGCGAGGCUGCCAGUCGUCGCACUCGAGCCGAGGCUGCAGGC
Figure 2.11: RNA EMSA of ESCRT-II with a panel of CLIP-tags containing the GA-rich motif
RNA EMSA of ESCRT-II ΔMBD and CLIP-tags from the gps2, znt2, and pigc transcripts. The sequences of the CLIP-tags (plus flanking regions on the transcript to total 40 nucleotides) are indicated with the GA-rich portions highlighted in black. ESCRT-II ΔMBD concentrations indicated by the white wedge are two-fold dilutions between 3.2µM -50nM.
Figure 2.12: Location of ESCRT-II CLIP-tags
Plot showing the distribution of ESCRT-II CLIP-tags across the length of transcripts. Each transcript was divided into 5’ UTR, coding sequence (CDS), and 3’ UTR regions, and each region was divided into bins spanning 10% of its total length. The location of the first nucleotide of each CLIP-tag, dinucleotide motif, or mononucleotide motif was mapped to these bins, and the percentage of sequences in each bin for each category was plotted.

Discussion

Previously, a novel role of ESCRT-II in RNA regulation was discovered in the localization of bicoid RNA in Drosophila oocytes (Irion and St Johnston, 2007). In this study, we expanded upon this observation to find that the interaction between ESCRT-II and RNA is conserved in an additional organism, Xenopus laevis. We took advantage of the high concentration of ESCRT-II in this system to further elucidate the molecular details of the ESCRT-II/RNA interaction.

We found that ESCRT-II interacts directly with RNA through Vps25 in Xenopus egg extracts. This finding contradicted the proposed model for the interaction between ESCRT-II and
bicoid RNA, which was suggested to occur through the Vps36 GLUE domain. The finding that the GLUE domain directly binds to bicoid RNA was based on the results of a similar in vitro UV-crosslinking approach as was used in our study (Irion and St Johnston, 2007). However, while we could observe RNA binding through the membrane binding domains of Vps22 and Vps36 in vitro, we failed to observe binding between Vps22 or Vps36 and RNA in Xenopus eggs. When the membrane binding domains of ESCRT-II are removed, the interaction between recombinant ESCRT-II and RNA is specific to ESCRT-II CLIP-tags and mimics the observed mechanism of binding in egg extract. These findings raise the possibility that the interaction between bicoid RNA and the Vps36 GLUE domain may not be physiologically relevant. However, Irion and colleagues were able to demonstrate a selective interaction between Vps36 and stem loop V of bicoid RNA, and the possibility remains that ESCRT-II could interact with bicoid RNA through a different mechanism than it does with transcripts in Xenopus eggs. Our finding that Vps25 is responsible for RNA binding in Xenopus eggs presents the possibility that ESCRT-II could interact with membranes and RNA simultaneously, giving ESCRT-II the potential to serve as a tether between the two.

The finding that Vps25 interacts directly with RNA targets in Xenopus eggs raises the question of where on Vps25, and which residues, are responsible for RNA binding. The crystal structures of ESCRT-II revealed that Vps25, as well as the other subunits of ESCRT-II, contain winged-helix domains (Hierro et al., 2004; Im and Hurley, 2008; Teo et al., 2004). While winged-helix domains have been largely characterized on transcription factors to mediate interactions with DNA, some RNA-binding proteins contain winged-helix domains as well, including La proteins and the translation elongation factor, SelB (reviewed in Harami et al., 2013). Given that Vps25 is composed almost entirely of winged-helix domains, it is likely that
this structural motif is responsible for RNA binding by ESCRT-II. While there is little sequence homology between Vps25 and the other ESCRT-II subunits, the structure of the winged-helix domains of all of the ESCRT-II subunits superimpose each other well, with a root mean square deviation of less than 2 Å (Hierro et al., 2004; Im and Hurley, 2008; Teo et al., 2004). The lack of RNA binding through the additional ESCRT-II subunits in egg extract through the winged-helix domains may be due to the position of those domains in the complex, resulting in a lack of exposure to RNA, or might suggest that RNA binding through Vps25 is influenced by the primary sequence of this subunit.

While Vps25 contains two winged-helix domains, the approach of this study could not distinguish between RNA binding by one domain versus the other. The exposure of the C-terminal winged-helix domain of Vps25 on the structure of ESCRT-II, with its only other known interacting factor being Vps20 of ESCRT-III (Im et al., 2009; Langelier et al., 2006), makes this portion of Vps25 a good candidate to consider as the RNA-binding domain of ESCRT-II. Future studies that further narrow down the residues responsible for RNA binding by ESCRT-II will provide important tools to further understand the role of ESCRT-II in RNA regulation. For example, the use of mutants of ESCRT-II that abolish RNA binding while preserving ESCRT-II’s function in endosome maturation could be used to tease apart these two distinct roles of ESCRT-II.

Analysis of the directly bound RNA targets of ESCRT-II revealed that many ESCRT-II targets have a long purine-rich motif. We found by RIP-seq that ESCRT-II has a selective interaction with RNAs, and our data suggest that this selective interaction may be achieved in part through recognition of the GA-rich motif. Specificity of ESCRT-II for the GA-rich motif could be demonstrated \textit{in vitro} using two independent RNA-binding assays. In the first assay, we
used a UV-crosslinking approach to reveal that Vps25 binds directly to a GA-rich CLIP-tag of ESCRT-II, but binding through Vps25 could not be detected to a negative control sequence in this assay. In addition, we found that after removing the membrane binding domains of Vps22 and Vps36, these other ESCRT-II subunits fail to bind to the GA-rich CLIP-tag in vitro. Similarly, we found by RNA EMSA that the ESCRT-II complex preferentially binds to GA-rich CLIP-tags in vitro. Thus, our molecular characterization of ESCRT-II/RNA binding in vitro agrees with our analysis of ESCRT-II/RNA interactions in egg extract, supporting a model in which Vps25 interacts with RNA targets by recognition of a purine-rich motif. Furthermore, these in vitro binding assays were performed with recombinant human ESCRT-II, suggesting that ESCRT-II’s RNA-binding properties are conserved in humans.

While our in vitro data suggests that the presence of a GA-rich motif is sufficient for binding by ESCRT-II, additional experiments are needed to address the necessity of the GA-rich motif for ESCRT-II binding. The fact that the GA-rich motif is not present in all of our CLIP-tags suggests that ESCRT-II could recognize RNAs through additional mechanisms. We identified an additional C-rich motif in our CLIP-seq library that is also enriched in ESCRT-II immunoprecipitations, but additional studies are needed to address the contribution of this motif to RNA binding by ESCRT-II.

While our CLIP-seq library is far from comprehensive, our RIP-seq library suggests putative targets of ESCRT-II through a genome-wide analysis. Gene ontology of the ESCRT-II-associated RNAs revealed an enrichment of particular classes of transcripts, namely transcripts encoding proteins involved with membranes, cell adhesion, cell motility, and the cytoskeleton. These classes are consistent with transcripts that typically undergo subcellular localization prior to translation. Interestingly, we isolated ESCRT-II under conditions in which ESCRT-II is
primarily cytoplasmic; thus, if these transcripts undergo localization, it is likely that they associate with ESCRT-II prior to their eventual localization in the cell. Whether or not ESCRT-II is involved in the subcellular localization of these transcripts in *Xenopus* remains to be investigated, and is addressed in preliminary experiments presented in Chapter 3.

**Conclusion**

In this study, we uncovered the molecular mechanism for the interaction between ESCRT-II and its RNA targets. We identified many direct RNA targets as well as the sequence specificity of ESCRT-II using CLIP-seq, and we revealed additional putative targets of ESCRT-II by RIP-seq. Additional studies are needed to address the role that ESCRT-II plays in RNA regulation. Potential RNA regulatory roles of ESCRT-II in RNA localization, splicing, and stability are addressed in Chapter 3, and additional possible roles are discussed in Chapter 4.
Chapter 3: A potential role of ESCRT-II in localizing RNAs to the mitotic spindle in *Xenopus laevis* eggs

Attributions

The preparation of the spindle RNA library was performed by Amy Emerman, Caterina Schweidenback, and Ashwini Jambhekar. While these data did not make it into this chapter, Elijah Carrier performed critical initial experiments addressing ESCRT-II/RNA interactions in HeLa cells. All other experiments in this chapter were performed by Amy Emerman. Mike Blower performed all bioinformatics analyses. All members of the Blower lab offered critical advice throughout this project.

Introduction

During cell division, proper segregation of chromosomes is achieved by the action of a macromolecular complex called the mitotic spindle. In general, the mitotic spindle is formed when microtubules emanating from two centrosomes attach to kinetochores to align and segregate chromosomes, and a variety of factors interact with the spindle to regulate its function (reviewed in Gadde and Heald, 2004).

Recently the mitotic spindle has been highlighted as a subcellular structure to which many RNAs localize. *In situ* hybridization approaches revealed that cyclinB1, Xbub3, xkid, and tpx2 mRNAs localize to the mitotic spindle in *Xenopus* oocytes (Eliscovich et al., 2008; Groisman et al., 2000). In addition, RNA has been detected on the centrosome in several organisms, including surf clams, snails, and flies (Alliegro and Alliegro, 2008; Alliegro et al., 2006; Kingsley et al., 2007; Lambert and Nagy, 2002; Lecuyer et al., 2007; Raff et al., 1990).
Furthermore, in genome-wide studies, taxol-stabilized microtubules from meiotic *Xenopus* egg extracts, which were used as a proxy for the meiotic spindle, were found to be associated with hundreds of mRNAs (Blower et al., 2007; Sharp et al., 2011). Localization of RNAs to the spindle may occur for a variety of reasons. Some mRNAs locally translate proteins at the spindle (Eliscovich et al., 2008; Groisman et al., 2000), some RNAs localize to the spindle to be asymmetrically sorted during cell division (Kingsley et al., 2007; Lambert and Nagy, 2002), and some play a noncoding role in spindle formation (Blower et al., 2005; Ideue et al., 2014; Jambhekar et al., 2014; Rosic et al., 2014).

In the previous chapter, we found that ESCRT-II associates with a class of RNAs that code for cytoskeleton- and centrosome-associated proteins. This result was particularly interesting given that ESCRT complexes have been found to associate with centrosomes and to impact centrosome number and spindle morphology (Frost et al., 2012; Jin et al., 2005; Langelier et al., 2006; Morita et al., 2010; Xie et al., 1998). ESCRT-II, in particular, has been reported to reside on the centrosome in COS7 cells and human osteosarcoma cells (Langelier et al., 2006), as well as on the spindle pole body (the yeast equivalent of the centrosome) in *S. pombe* (Jin et al., 2005). However, the precise roles that ESCRT complexes play on the centrosome remain unclear.

In this study, we examined whether ESCRT-II interacts with the same RNAs that are on the mitotic spindle, and we began to address the role that ESCRT-II plays in the regulation of these RNAs. We began this study by first generating a new library of spindle-associated RNAs from fully-formed mitotic spindles. We found that ESCRT-II interacts with RNAs that are highly enriched on the mitotic spindle, and we obtained preliminary evidence that ESCRT-II may affect the localization of RNAs to the spindle. In addition, we addressed two other potential roles of
ESCRT-II in RNA regulation: whether ESCRT-II can impact alternative splicing as a precursor to its role in the cytoplasm and if ESCRT-II plays a role in RNA stability. However, we found no evidence to support a model in which ESCRT-II influences either splicing or stability of its RNA targets.

Results

ESCRT-II localized to centrosomes in *Xenopus* Xl177 cells and to exogenous centrosomes added to egg extract

In Chapter 2, we found that ESCRT-II associates with transcripts encoding proteins that interact with the cytoskeleton. These findings were particularly interesting in light of previous studies demonstrating that ESCRT-II, as well as other ESCRT complexes, localize to centrosomes in a variety of cell types, including COS7 cells and yeast (Jin et al., 2005; Langelier et al., 2006). To determine if ESCRT-II associates with the centrosome in *Xenopus* cells, we performed immunofluorescence for ESCRT-II on Xl177 cells, a *Xenopus* tadpole cell line. ESCRT-II colocalizes with puncta that are positive for γ-tubulin, indicative of centrosomal localization (Figure 3.1A). Furthermore, ESCRT-II displays this localization pattern in both interphase and mitotic cells. Therefore, we conclude that ESCRT-II localizes to the centrosome in *Xenopus* Xl177 cells throughout the cell cycle.

In contrast, we failed to convincingly observe ESCRT-II by immunofluorescence on fully-formed spindles in *Xenopus* egg extract (not shown). Spindle formation in egg extract is initiated by the addition of *Xenopus* sperm nuclei to the extract, and each sperm nucleus is associated with a centrosome. We asked if ESCRT-II interacts with these exogenously added centrosomes in egg extract prior to spindle formation. We incubated the sperm nuclei and their
Figure 3.1: ESCRT-II resides on the centrosome in Xenopus Xl177 cells and on sperm nuclei in egg extract
A) Immunofluorescence for ESCRT-II in Xenopus Xl177 cells, demonstrating colocalization with the centrosomal marker γ-tubulin in both interphase and mitotic cells. B) Immunofluorescence for ESCRT-II on sperm nuclei after incubation in Xenopus egg extract, demonstrating colocalization on the sperm-associated centrosome with the centrosomal marker ε-tubulin. A and B) Scale bar = 5µm.

associated centrosomes in extract, then performed immunofluorescence for ESCRT-II or ε-tubulin (a marker of the centrosome) on pelleted nuclei and centrosomes. We found that ESCRT-II and ε-tubulin colocalize at a single puncta associated with the sperm nuclei (Figure 3.1B). Taken together, these data suggest that ESCRT-II interacts with the centrosomes in egg extract initially, but does not associate with fully-formed spindles.
Generation of a library of mitotic spindle-associated RNAs

Given that ESCRT-II localizes to centrosomes and associates with a category of RNAs that encode cytoskeleton and centrosomal proteins, we asked if ESCRT-II interacts with RNAs that are associated with the mitotic spindle. The previously available genome-wide libraries of spindle-associated RNAs were inferred by sequencing mRNA associated with taxol-stabilized microtubules formed in meiotic egg extract. Because ESCRT-II associates with the centrosome, these libraries are not likely to be enriched for ESCRT-II-associated RNAs, as taxol-stabilized microtubules lack centrosomes. Therefore, to investigate the association between ESCRT-II-associated RNAs and spindle-localized RNAs, we first generated a library of spindle-associated RNAs from fully-formed mitotic spindles.

We first optimized the conditions for isolating mitotic spindles. Methods to prepare mitotic spindles in *Xenopus* egg extracts around exogenously added *Xenopus* sperm nuclei are well-established (Hannak and Heald, 2006). We followed these methods and optimized the spindle pelleting conditions to purify spindle-associated RNA. We monitored the stringency of the pelleting conditions by assaying for pelleting of additional cellular components by Western blot. A 40% glycerol cushion pellets the endoplasmic reticulum (ER) independently of the spindle (when microtubules are destabilized with nocodazole) and is therefore not stringent enough for isolating spindle-associated RNAs (Figure 3.2A). In contrast, the ER copellets with the spindle through a 60% glycerol cushion, but does not pellet independently of the spindle (Figure 3.2A). We chose to generate spindle-associated RNA libraries from samples that were first treated with 0.5% Triton X-100 to disrupt membranes prior to its application to a 60% glycerol cushion. RNA copellets with the spindle under these conditions, but not when microtubules are destabilized with nocodazole (Figure 3.2B). Under these conditions,
Figure 3.2: Optimization of spindle-pelleting conditions for the generation of a spindle RNA library

A) Mitotic spindles were formed in *Xenopus* egg extract around exogenously added sperm nuclei as previously described (Hannak and Heald, 2006). The spindle-containing extract was diluted in 30% glycerol/1X BrB80 with or without 0.5% Triton X-100, then loaded onto a 5 mL, 40% glycerol/1X BrB80 cushion or a 10 mL, 60% glycerol/1X BrB80 cushion for centrifugation. The resulting pellets were analyzed by Western blot for the ESCRT-II subunit Vps25 or for markers of the mitotic spindle (α-tubulin) or the endoplasmic reticulum (trap-α). Nocodazole (Noc) was used as a control to inhibit spindle formation.

B) Mitotic spindles were formed as in (A), then following dilution with 30% glycerol/1X BrB80 with 0.5% Triton X-100 and isolation by centrifugation through a 60% glycerol/1X BrB80, 10mL cushion, RNA was isolated from the pellet and analyzed on an agarose gel.
markers for the ER no longer pellet with spindles (Figure 3.2A), and therefore the RNA library will consist of RNAs that interact with the spindle independently of membranous organelles. Similar to results obtained by immunofluorescence (above), we found that ESCRT-II does not copellet with fully-formed mitotic spindles when assayed by Western blot (Figure 3.2A).

We generated a library of the spindle-associated RNAs and submitted these transcripts for high-throughput sequencing. We found that the spindle RNA library has a median spindle-enrichment value of 0.96 and a mean spindle-enrichment value of 1.60, and that 439 mRNAs are at least 4-fold enriched on the spindle (Figure 3.3A). In addition, we observed a decent correlation of spindle enrichment of RNAs across biological replicates (r=0.83), although some variability exists (Figure 3.3B). We validated the spindle library by qPCR and found that RNAs predicted to be enriched by the sequencing library are more enriched on spindles compared to RNAs that have previously been described as spindle-localized (Groisman et al., 2000). Furthermore, RNAs that were predicted to be underrepresented in the spindle sequencing library are also underenriched when assayed by qPCR (Figure 3.3C).

To determine if specific classes of RNAs are enriched on the spindle, we performed a gene ontology analysis of the spindle-associated RNA library (Huang da et al., 2009a, b). We found that spindle-associated RNAs encode proteins involved with the cytoskeleton, actin binding, nucleotide binding, microtubule motors, and the centrosome (Table 3.1). These categories are similar to those observed for RNAs enriched on taxol-stabilized microtubules from Xenopus meiotic egg extracts (Blower et al., 2007; Sharp et al., 2011). Given that the proteins encoded by these categories are expected to associate with the mitotic spindle, these data are
Figure 3.3: Library of spindle enriched RNAs

A and B) Mitotic spindles were formed in *Xenopus* egg extract and isolated through a 10mL, 60% glycerol/1XBrB80 cushion in the presence of 0.5% Triton X-100. The associated RNAs were isolated and sequenced by high-throughput sequencing. A) Scatterplot of transcript abundance (RPKM) on isolated mitotic spindles compared to total egg RNA. Red circles indicate transcripts that are highly enriched on the mitotic spindle relative to their abundance in total egg extract (at least 4-fold enriched). All genes with a combined read count of >100 in the spindle and total RNA libraries are shown. Blue line indicates equal RPKM in both samples. B) Correlation of spindle enrichment (RPKM in spindle/total RNA samples) across two biological replicates. The Pearson correlation coefficient is 0.83. C) qPCR validation of enrichment on spindles isolated as in (A and B) of mRNAs predicted by high-throughput sequencing to be spindle-enriched compared to positive control mRNAs previously characterized as spindle-localized (Groisman et al., 2000) and negative control mRNAs predicted by high-throughput sequencing to be underenriched on spindles. qPCRs for spindle enrichment of akap9, pcnt, ppdpf, and sec61β have been repeated in >6 independent experiments and similar results were observed.
Table 3.1: Gene ontology analysis of the spindle RNA library

Summary of the top annotation clusters in overrepresented and underrepresented transcripts in the spindle RNA library, identified by NCBI DAVID (Huang da et al., 2009a, b).

<table>
<thead>
<tr>
<th>Annotation cluster</th>
<th>Enrichment score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overrepresented clusters:</strong></td>
<td></td>
</tr>
<tr>
<td>Cytoskeleton/non membrane bound organelle</td>
<td>15.95</td>
</tr>
<tr>
<td>Cytoskeleton/Microtubule</td>
<td>10.58</td>
</tr>
<tr>
<td>Actin binding</td>
<td>5.13</td>
</tr>
<tr>
<td>Nucleotide binding</td>
<td>4.72</td>
</tr>
<tr>
<td>Microtubule motor proteins</td>
<td>3.84</td>
</tr>
<tr>
<td>Microtubule cytoskeleton/centrosome</td>
<td>3.17</td>
</tr>
<tr>
<td><strong>Underrepresented clusters:</strong></td>
<td></td>
</tr>
<tr>
<td>CS domain</td>
<td>2.11</td>
</tr>
<tr>
<td>MAPK signaling pathway</td>
<td>1.84</td>
</tr>
<tr>
<td>SNARE complex</td>
<td>1.69</td>
</tr>
</tbody>
</table>

consistent with the possibility that RNAs associate with the mitotic spindle to undergo localized translation.

ESCRT-II associates with RNAs that are enriched on the mitotic spindle

To determine if ESCRT-II interacts with RNAs that associate with the mitotic spindle, we compared our ESCRT-II RIP-seq library (see Chapter 2) to the spindle RNA library. Among the transcripts that are both highly spindle-enriched and highly ESCRT-II-enriched, we observed a striking correlation between these two datasets (Figure 3.4). In contrast, no association was observed between the two datasets for transcripts that have low spindle-enrichment or ESCRT-II-enrichment values (Figure 3.4). Taking together the gene ontology of the ESCRT-II RIP-seq
Figure 3.4: Correlation of ESCRT-II RIP-seq and spindle RNA libraries
Scatterplot demonstrating a correlation between enrichment of transcripts in ESCRT-II immunoprecipitations (RPKM in ESCRT-II IP/Total) and enrichment on mitotic spindles (RPKM in Spindle/Total). Blue line indicates equal enrichment.

library and the correlation between the ESCRT-II RIP-seq library and the spindle RNA library, we conclude that ESCRT-II interacts with highly spindle-enriched RNAs. Given the fact that ESCRT-II does not localize to fully-formed spindles, any regulation of these RNAs by ESCRT-II would likely occur before the full spindle is formed; for example, RNA regulation could occur during initial stages of spindle formation when ESCRT-II interacts with the exogenously added centrosome.

Ongoing experiments to determine if ESCRT-II affects RNA localization to the spindle

Our data suggest that ESCRT-II interacts with highly spindle-enriched RNAs, and that ESCRT-II associates with centrosomes early in spindle formation in egg extract. ESCRT-II
could play a variety of roles in regulating these RNAs. Given that ESCRT-II is involved in RNA localization in *Drosophila* oocytes (Irion and St Johnston, 2007), we hypothesized that ESCRT-II may be involved in localizing RNAs to the mitotic spindle.

To address this possibility, we isolated spindle-associated RNAs under two conditions, one in which ESCRT-II was depleted from egg extract and another in which the extract underwent a mock depletion with nonspecific rabbit IgG. We performed this experiment in duplicate and submitted these RNA samples for high-throughput sequencing. We compared spindle enrichment of transcripts in mock-depleted extracts to spindle enrichment in ESCRT-II-depleted extracts. We found that many mRNAs are less spindle enriched with ESCRT-II depletion and that mRNAs that are enriched in the ESCRT-II RIP-seq library in particular tend to show decreases in spindle enrichment with ESCRT-II depletion (Figure 3.5). However, the degree of this decrease in spindle enrichment with ESCRT-II depletion varied across two biological replicates (Figure 3.5).

While preliminary, these data raise the possibility that ESCRT-II could impact RNA localization in additional systems beyond *Drosophila* oocytes. We are in the process of validating these data by qPCR and introducing additional controls. In particular, it will be important to determine if recombinant ESCRT-II can rescue the effect on spindle enrichment and if depletion of additional ESCRT complexes also affect spindle localization, or if this effect is specific to ESCRT-II.

**ESCRT-II does not affect splicing or stability of transcripts**

Many *trans*-acting factors in RNA localization pathways play additional roles in other forms of RNA regulation (reviewed in Martin and Ephrussi, 2009). In some cases, *trans*-acting
Figure 3.5: Spindle enrichment in ESCRT-II depleted extracts
ESCRT-II was immunodepleted from egg extract or a mock immunodepletion was performed, then mitotic spindles were formed and isolated through a 10mL, 60% glycerol/1XBrB80 cushion in the presence of 0.5% Triton X-100. The spindle-associated RNAs from each sample were isolated and sequenced by high-throughput sequencing. Scatterplots from two biological replicates are shown demonstrating differences in spindle enrichment of transcripts in mock-versus ESCRT-II-depleted extracts. The blue line indicates equal enrichment in the two samples. Red circles are transcripts that are enriched in ESCRT-II immunoprecipitations (top 100 enriched transcripts). Depletion of ESCRT-II from the extracts was confirmed by Western blot for Vps25.
factors have been found to play dual roles in splicing and RNA localization (reviewed in Martin and Ephrussi, 2009). ESCRT-II has been found to play a poorly defined function in the nucleus where it interacts with the elongation factor, ELL (Kamura et al., 2001; Schmidt et al., 1999; Shilatifard, 1998), suggesting the possibility that ESCRT-II could first interact with pre-mRNAs in the nucleus. Given the connections between splicing and RNA localization and the nuclear localization of ESCRT-II, we hypothesized that ESCRT-II may be involved in RNA splicing. Such an effect could occur through interactions between ESCRT-II and purine-rich motifs on the pre-mRNA, which can act as splicing enhancers on some transcripts (reviewed in Matlin et al., 2005), and which we found in Chapter 2 are recognized by ESCRT-II. In addition to a role in splicing, we sought to determine if ESCRT-II plays a role in regulating the stability of transcripts. Because these experiments required newly transcribed RNA, they could not be performed in Xenopus eggs, which are transcriptionally silent. Therefore, we turned to tissue culture systems to address these questions.

Initially, we attempted to perform these experiments in Xenopus Xl177 cells, as this is the same organism that our RIP-seq and CLIP-seq datasets are from but at a different stage in development. To perform knockdowns in this cell line, we generated endoribonuclease-prepared siRNAs (esiRNAs) (Yang et al., 2002; Yang et al., 2004) against Vps25. We found that cotransfection with an Ago2 plasmid was necessary for the esiRNAs to have any effect on Vps25 mRNA levels, consistent with previous reports that Ago2 is limiting in early stages of Xenopus development (Chen et al., 2009; Lund et al., 2011). However, while Ago2 cotransfection improved the knockdown efficiency, we could still only achieve about a 59% knockdown of the Vps25 mRNA (as determined by qPCR) and about a 50% knockdown of the Vps25 protein.
Figure 3.6: siRNA knockdowns of Vps25 in Xenopus Xl77 and HeLa cells

A) Western blot of Vps25 in Xl177 cells 3 days after transfection with GFP or Vps25 esiRNAs. A similar amount of remaining protein was observed 5 days post-transfection (not shown). B) Western blot of Vps25 in dilutions of HeLa cell lysates 4 days post-transfection with commercial Vps25 and negative control siRNAs or homemade Vps25 or GFP esiRNAs. α-tubulin is shown as a loading control.

(Figure 3.6A). This level of knockdown would not be sufficient to analyze differential transcript or splice variant abundance, so we chose not to use Xl177 cells for these experiments.

We instead used HeLa cells to explore a possible function of ESCRT-II in RNA stability or splicing, as these cells have a high transfection efficiency and a well-annotated genome. We performed knockdowns in HeLa cells with either a commercial siRNA against Vps25 or a homemade esiRNA. We were able to achieve a 96% knockdown of Vps25 mRNA (as measured by qPCR) and approximately 70-95% knockdown of Vps25 protein using either siRNA (Figure
We isolated RNA from HeLa cells following Vps25 knockdowns using each siRNA individually (or the corresponding negative control siRNA) and generated cDNA libraries for high-throughput sequencing.

To assay for changes in stability, we compared the change in abundance of transcripts with Vps25 knockdown versus the corresponding negative control across the two different siRNA approaches. We did not observe any obvious reproducible changes in abundance of transcripts, and slight changes in abundance across the two datasets are likely within the noise of the experiment (Figure 3.7). Therefore, we concluded that ESCRT-II does not affect RNA stability in HeLa cells.

We used the program MATS (Park et al., 2013; Shen et al., 2012) to assay for changes in splicing with ESCRT-II knockdown. MATS identified 5 transcripts that reproducibly had differential exon inclusion between Vps25 knockdown and control samples across the two knockdown approaches ($P$-value < $2 \times 10^{-4}$ and false discovery rate < 0.16). In contrast, MATS detected hundreds of changes in splicing with overexpression of a true splicing factor (Shen et al., 2012). Follow-up by RT-PCR demonstrated that these predicted 5 changes were in fact false positives (Figure 3.8A). In contrast, one of our negative control siRNAs appeared to have knocked down a splicing factor, and we found changes between the negative control knockdown and non-transfected cells that could be validated by RT-PCR (Figure 3.8B). We therefore conclude that ESCRT-II does not impact splicing in HeLa cells.
Figure 3.7: ESCRT-II does not affect RNA abundance in HeLa cells
Scatterplot depicting the change in abundance of transcripts with Vps25 knockdown across two independent siRNAs and controls. The change in abundance of the Vps25 transcript is highlighted in red for reference.
Figure 3.8: ESCRT-II does not affect RNA splicing in HeLa cells
A) RT-PCR of transcripts predicted by MATs (Park et al., 2013; Shen et al., 2012) to have differential splicing with Vps25 knockdown. Differential splicing was analyzed in Vps28 knockdowns (a subunit of ESCRT-I) in addition to GFP and Vps25 knockdowns. B) RT-PCR of a transcript (cd47) that was predicted by MATs to have differential splicing following knockdown with the negative control siRNA from Ambion compared to nontransfected cells. A-B) Primers were designed to exons flanking the predicted skipped exons, such that a longer PCR product indicates inclusion of the skipped exon.
Discussion

In this study, we generated an improved library of spindle-associated RNAs by isolating RNAs that copurify with fully-formed spindles. Unlike libraries generated from RNAs associated with taxol-stabilized microtubules from meiotic egg extracts, these libraries may also include transcripts that are associated with centrosomes and chromatin in addition to microtubules. Further analysis and comparison of these libraries may reveal novel spindle-enriched transcripts that involve these additional components of the spindle. In addition, studies investigating individual transcripts on the spindle will be important to further understand the significance of RNA localization to the spindle. A major category of spindle-localized RNAs are those that code for cytoskeletal proteins, and given previous reports that ribosomes associate with the spindle in egg extracts (Blower et al., 2007; Mitchison et al., 2004), these data suggest that localized translation may be one important outcome of RNA localization to the spindle.

We found that highly spindle-associated RNAs show a striking correlation with highly ESCRT-II-associated RNAs. Whether or not ESCRT-II plays a role in regulating these RNAs on the spindle is unclear. Previous studies found that mutations in the fission yeast homologue of the ESCRT-II subunit, Vps22, result in oversized and overamplified spindle pole bodies (the yeast equivalent of the centrosome) (Jin et al., 2005). Given the growing body of evidence that unique populations of RNAs exist on centrosomes (Alliegro and Alliegro, 2008; Alliegro et al., 2006; Kingsley et al., 2007; Lambert and Nagy, 2002; Lecuyer et al., 2007; Raff et al., 1990), it will be important to further investigate whether ESCRT-II regulation of RNAs on the centrosome contributes to the effect of ESCRT-II on centrosome maturation and whether such an effect is conserved across organisms.
We obtained preliminary evidence that ESCRT-II might impact RNA localization to the mitotic spindle. Specifically, we found that the bulk of RNAs are equally represented on the spindle in ESCRT-II- and mock-depleted egg extracts, but that highly spindle-enriched RNAs show a decrease in spindle enrichment with ESCRT-II depletion. However, in a biological replicate of this experiment, this difference was less striking. Experiments are currently ongoing to determine if individual transcripts are reproducibly reduced on the spindle with ESCRT-II depletion and if addition of recombinant ESCRT-II can rescue this phenotype. In addition, experiments in which additional ESCRT complexes are depleted will be important to determine if this phenotype is independent of ESCRT-II’s role in endosome maturation.

Of note, the phenotype in an ESCRT-II-depleted egg extract is only a partial decrease in RNA enrichment on the spindle. This finding resembles that of ESCRT-II mutations in *Drosophila* oocytes, where the impact on *bicoid* localization is only partially impaired (Irion and St Johnston, 2007). In the case of *bicoid* mRNA, this partial phenotype indicates that ESCRT-II works in concert with additional factors that act at multiple stages to influence the localization of *bicoid*, and many of the additional *trans*-acting factors involved in *bicoid* localization have been identified (reviewed in Weil, 2014). If ESCRT-II plays a role in localizing RNAs to the spindle in *Xenopus* eggs, this pathway is likely to involve several additional *trans*-acting factors.

We observed that ESCRT-II localizes to the centrosome in *Xenopus* XI177 cells as well as on sperm nuclei-associated centrosomes that are introduced into *Xenopus* egg extracts, consistent with previous reports that ESCRT-II resides on centrosomes (Jin et al., 2005; Langelier et al., 2006). However, we failed to observe ESCRT-II on fully-formed *Xenopus* mitotic spindles. These spindles somewhat resemble meiotic spindles, and this difference could account for the differences observed in the localization of ESCRT-II in XI177 cells and egg
extract spindles. The observation that ESCRT-II can interact with the sperm centrosome in egg extracts suggests that ESCRT-II may be present during initial stages of spindle formation and could influence spindle RNAs at this point. However, additional studies are required to confirm this possibility. The fact that ESCRT-II fails to localize to fully-formed spindles raises the question of how ESCRT-II could be involved in localizing RNAs to the spindle. A potential model is that ESCRT-II could interact with spindles during their initial formation, then transfer RNAs to other factors that could anchor the RNAs on the spindle.

We investigated two other potential roles of ESCRT-II in RNA regulation. The association between ESCRT-II and a transcription elongation factor (Kamura et al., 2001; Schmidt et al., 1999; Shilatifard, 1998) offers evidence that ESCRT-II could interact with pre-mRNAs following transcription. Thus, we investigated whether ESCRT-II impacts the processing of pre-mRNAs in the nucleus. Specifically, we asked if ESCRT-II impacts RNA splicing, as this process is known to influence the subcellular localization of some transcripts (reviewed in Martin and Ephrussi, 2009). However, we found no evidence to suggest that ESCRT-II is involved in RNA splicing. Furthermore, we found that the stability of transcripts is not impacted by ESCRT-II depletion. One caveat to these experiments is that they were performed in HeLa cells, and while recombinant human ESCRT-II is capable of interacting with RNAs in vitro, we do not know if the same interactions occur in vivo. Unfortunately, because these experiments required a system in which new transcription occurs, we could not address the impact of ESCRT-II on splicing or RNA stability in Xenopus eggs, the system in which we have discovered an in vivo interaction between ESCRT-II and RNA.
Conclusion

In this study we generated an improved genome-wide library of transcripts associated with the mitotic spindle. We found that highly ESCRT-II-associated RNAs correlate with highly spindle-associated RNAs, and we found preliminary evidence that ESCRT-II might influence RNA localization to the mitotic spindle. However, additional studies are needed to fully understand the impact that ESCRT-II has on spindle-localized RNAs.
Chapter 4: Conclusions, Future Directions, and Broad Implications

Summary of results and implications in RNA localization

In 2007, an exciting finding was published that implicated ESCRT-II in the localization of *bicoid* mRNA (Irion and St Johnston, 2007). Though ESCRT-II was not previously known to be involved in RNA regulation, it had a well-established role in receptor downregulation and endosome maturation (reviewed in Hurley, 2008). The discovery that ESCRT-II had an RNA regulatory role provided an early link between two very distinct areas of biology: RNA regulation and membrane trafficking. Meanwhile, several additional studies provided links between endosomes and RNA regulation. In particular, the finding that exosomes contain selective subsets of RNAs (Valadi et al., 2007) and that miRNA-mediated silencing occurs on endosomes (Gibbings et al., 2009; Lee et al., 2009) suggested that RNAs directly associate with endosomes and are selectively trafficked to these organelles. Furthermore, the finding that mRNAs can traffic on endosomes to undergo long distance transport in fungal cells raised the question of whether such a mechanism might occur in higher organisms (Baumann et al., 2014; Baumann et al., 2012). With ESCRT-II intimately involved in endosome biology, further studies were needed to understand whether the role of ESCRT-II in RNA regulation is conserved, if ESCRT-II could impact additional areas of RNA regulation beyond localization, and if any of these RNA regulatory events are related to ESCRT-II’s role in endosome biology.

This thesis presents work that advances the understanding of ESCRT-II in RNA regulation. Specifically, we discovered that ESCRT-II interacts with hundreds of RNAs in *Xenopus* eggs, that this interaction is mediated through the ESCRT-II subunit Vps25, and that ESCRT-II recognizes its RNA targets through a polypurine motif. Our finding that ESCRT-II
interacts with RNAs through Vps25 provides a mechanism by which ESCRT-II could bind to membranes and RNA simultaneously. By uncovering the molecular details of the ESCRT-II/RNA interaction, we now have tools that can be used to further investigate the impact that ESCRT-II has on RNA regulation. In addition, our findings add to the growing list of conserved factors involved in RNA regulation.

The gene ontology categories of RNAs associated with ESCRT-II suggest that ESCRT-II may associate with RNAs that undergo localization in the cell. These categories include RNAs encoding proteins involved in cell migration, a process known to involve localized RNA (reviewed in Medioni et al., 2012), and the cytoskeleton and the centrosome, subcellular structures to which many mRNAs localize (Alliegro and Alliegro, 2008; Alliegro et al., 2006; Blower et al., 2007; Eliscovich et al., 2008; Groisman et al., 2000; Kingsley et al., 2007; Lambert and Nagy, 2002; Lecuyer et al., 2007; Raff et al., 1990; Sharp et al., 2011). Furthermore, we found that ESCRT-II associates with mRNAs encoding membrane-associated proteins, which is particularly interesting given ESCRT-II’s transient interactions with membranes. However, ESCRT-II is primarily cytoplasmic in the cell lysate from which we performed the RNA immunoprecipitations, raising the possibility that ESCRT-II associates with its RNA targets prior to their localization in the cell. We investigated the possibility that ESCRT-II may be involved in the localization of a subset of its associated RNAs in Chapter 3, by providing preliminary evidence that ESCRT-II is involved in localizing RNAs to the mitotic spindle. Future studies should focus on the impact that ESCRT-II has on spindle-localized RNAs. In particular, it will be interesting to examine whether misregulation of centrosome-associated RNAs contributes to the defects observed in centrosome morphology in ESCRT-II mutant yeast cells (Jin et al., 2005).
ESCRT-II has several features that make it a potentially important factor for RNA localization. First, ESCRT-II is correctly positioned to interact with newly transcribed RNAs in the nucleus through an association with a transcription elongation factor (Kamura et al., 2001; Schmidt et al., 1999; Shilatifard, 1998). Many trans-acting factors for RNA localization pathways associate with transcripts in the nucleus and are exported as a ribonucleoprotein complex (RNP) into the cytoplasm, linking the nuclear history of the RNA with its cytoplasmic regulation (reviewed in Giorgi and Moore, 2007), and ESCRT-II could potentially provide this continuity. Second, ESCRT-II associates with the dynein/dynactin complex through an interaction with the protein, RILP (Jordens et al., 2001; Progida et al., 2006; Wang and Hong, 2006), providing a mechanism by which ESCRT-II could link RNAs to transport machinery for active transport to its destination. Finally, the transient interaction between ESCRT-II and endosomes could provide a pathway to target RNAs to endosomes, promoting the RNA to hitchhike on the endosome for long distance transport in the cell. Long distance RNA transport on endosomes has been observed in the fungus Ustilago maydis (Baumann et al., 2014; Baumann et al., 2012). Such a mechanism has not been observed in higher organisms, but neurons have a similarly arranged cytoskeleton and similar molecular motors involved in RNA transport as in Ustilago maydis, and will therefore be an important system in which to look for such modes of RNA transport (reviewed in Jansen et al., 2014; Steinberg and Perez-Martin, 2008). In addition, an association with endosomes might influence the trafficking of retroviral genomic RNA to the plasma membrane (Basyuk et al., 2003; Lehmann et al., 2009; Molle et al., 2009). However, these findings have been controversial, as others have found that retroviral genomic RNA reaches the plasma membrane primarily by diffusion (Chen et al., 2014). Of note, HIV genomic RNA accumulates at nuclear and juxtanuclear regions following ESCRT-II knockdown, but a
direct role of ESCRT-II in this pathway has not been determined (Ghoujal et al., 2012). Thus, the recently discovered roles of ESCRT-II in RNA localization pathways is supported by several previously identified features of this complex.

ESCRT-II CLIP-tags are generally found in the coding region of the transcript, suggesting that the ESCRT-II/RNA association should be lost once translation of the RNA begins. Therefore, ESCRT-II must associate with RNAs that are in a translationally repressed state, as is characteristic of many maternal mRNAs in *Xenopus* eggs (reviewed in Richter and Lasko, 2011). We favor a model in which ESCRT-II interacts with transcripts in the nucleus, and following export into the cytoplasm, the interaction between ESCRT-II and translationally repressed maternal mRNAs persists until later in development when translation of the mRNA begins. Such a mechanism could provide a means to release the RNA from an ESCRT-II-containing transport RNP upon localization and the pioneer round of translation. However, future experiments are needed to confirm that ESCRT-II does, in fact, interact with translationally repressed mRNAs.

An endosomal sorting complex as an RNA-binding factor

A major outstanding question is why would the cell repurpose an endosomal sorting complex as an RNA-binding factor? One possibility is that ESCRT-II could play completely separate roles in RNA regulation and membrane trafficking. Cases of such “moonlighting” proteins are widespread throughout biology and may arise to reduce the number of genes required in an organism or as a natural process in evolution to adapt existing proteins for new functions (reviewed in Huberts and van der Klei, 2010). However, a more exciting possibility is that ESCRT-II could provide a link between RNA regulation and endosome biology.
In particular, it would be interesting to determine if ESCRT-II is involved in the selective sorting of RNAs into exosomes. Addressing this question will require a better understanding of the molecular mechanisms that control the formation of exosomes, as well as the development of better tools for classifying and purifying extracellular vesicles. Exosomes are derived from MVBs, but these MVBs can be generated by both ESCRT-dependent and ESCRT-independent mechanisms (reviewed in Colombo et al., 2014). This concept is further highlighted by the fact that knockdown of ESCRT complexes do not completely abolish the release of exosomes, and not all members of the ESCRT pathway have the same effect on exosome release and size (Colombo et al., 2013). To complicate matters further, additional extracellular vesicles exist that are shed from the plasma membrane and are completely independent of MVBs (reviewed in Raposo and Stoorvogel, 2013). Therefore, when analyzing the RNA content of extracellular vesicles, many researchers analyze the RNA content of a heterogeneous population of vesicles, complicating the analysis and adding noise to the population of vesicles derived from any one particular source (reviewed in Thery, 2011). If it is discovered that RNAs are sorted into intraluminal vesicles that are formed in an ESCRT-dependent manner, then ESCRT-II will be an important complex to consider as a factor involved in this selective sorting, as ESCRT-II is positioned right at the membrane neck as intraluminal vesicles are forming. In addition, with improved purification protocols of ESCRT-dependent exosomes, it will be important to ask if GA-rich motifs are enriched in the exosomal RNA population. Of note, hnRNPA2B1 has been suggested to impact miRNA sorting into exosomes, and interestingly, a GA-rich motif was suggested to mediate this selective sorting (Villarroya-Beltri et al., 2013).

Similarly, it would be interesting to determine if ESCRT-II plays a role in the miRNA-mediated silencing that occurs on endosomes (Gibbings et al., 2009; Lee et al., 2009), perhaps by
localizing mRNAs to the endosome to be targeted by the RISC machinery. While it was found that knockdown of ESCRT-II impairs miRNA-mediated silencing, this finding may largely be due to a disruption of endosome maturation, as knockdowns in additional ESCRT complexes had the same effect (Gibbings et al., 2009). The possibility that a loss of ESCRT-II/RNA interactions also contributes to this phenotype could not be distinguished from an impact on endosome maturation. Mutations in ESCRT-II that specifically affect RNA binding but not endosome maturation could be used to further investigate this possibility.

Finally, it will be important to ask if ESCRT-II is involved in the translational regulation of mRNAs. Such a role would be particularly intriguing if coupled to receptor downregulation on endosomes, thereby coupling extracellular signaling with a translational output. Neurons are an interesting case in which extracellular cues lead to a localized, translational response, but few mechanisms to achieve localized translation in response to cell signaling have been identified (reviewed in Jung et al., 2014). In a recently discovered mechanism, the cell surface receptor DCC interacts with translation machinery to influence translation upon binding of its ligand, netrin (Tcherkezian et al., 2010). One could imagine an additional mechanism to couple extracellular signaling with a translational response, by stimulating ESCRT-II to release mRNAs in response to its recruitment to endosomes during receptor downregulation.

**Potential for RNA to impact endosome maturation**

Additionally, it would be interesting to address whether RNA impacts ESCRT-II’s activity at endosomes. For example, does RNA-binding prevent ESCRT-II from forming intraluminal vesicles? We found that ESCRT-II interacts with RNA through Vps25, the other binding partner of which is Vps20 of ESCRT-III (Im et al., 2009; Langelier et al., 2006). If RNA
could block this interaction, it may prevent endosomal maturation from occurring. Recently, giant unilamellar vesicles have been used to tease apart the mechanistic details of how ESCRT complexes interact with each other to form intraluminal vesicles (Im et al., 2009; Wollert and Hurley, 2010; Wollert et al., 2009). Similar experiments in which RNA is introduced could be used to determine if RNA influences the kinetics of intraluminal vesicle formation.

Conclusion

We have expanded the understanding of ESCRT-II in RNA regulation by demonstrating that ESCRT-II/RNA interactions are conserved across organisms and by elucidating the molecular details of the ESCRT-II/RNA interaction. Our findings further support the emerging links between endosomes and RNA regulation and suggest that ESCRT-II will be an important molecular player to consider in RNA localization and regulation pathways involving endosomes.
Chapter 5: Materials and Methods

Oocyte and egg extracts

_Xenopus_ egg extract was prepared as previously described (Hannak and Heald, 2006). _Xenopus_ oocyte lysate was prepared from freshly dissected oocytes that were separated from follicle cells with collagenase (C5138, Sigma) dissolved in 0.1M sodium phosphate pH 7.4. Following collagenase treatment, oocytes were washed in 1X Modified Barth’s Saline (88mM NaCl, 1mM KCl, 1mM MgSO4, 5mM Hepes, 2.5mM NaHCO3, and 0.7mM CaCl2, pH 7.8), then separated into stage 1-3 and stage 4-6 oocytes using a 450µM mesh screen. Stage 1-3 oocyte lysate was a gift from James Toombs and was prepared by washing oocytes with 1X XB (100mM KCl, 0.1mM CaCl2, 1mM MgCl2, 50mM sucrose, and 10mM Hepes, pH 7.7), Dounce homogenizing in the presence of protease inhibitors (leupeptin, pepstatin, and chymostatin) and cytochalasin D, centrifuging at 20,000 x g for 5 min at 4°C, then collecting the cytoplasmic layer. Stage 4-6 oocyte lysates were prepared similarly to egg extract (Hannak and Heald, 2006), except without cytochalasin D, and the oocytes were homogenized prior to centrifugation. _Xenopus_ egg light membrane and cytosol fractions that were used to assay for localization of ESCRT-II were a gift from Dianne Schwarz and were prepared as previously described (Schwarz and Blower, 2014).

Recombinant proteins

An expression construct of the ESCRT-II complex was generated by cloning the open reading frames of _Xenopus laevis_ Vps22 (IMAGE clone: 7197421), Vps36 (IMAGE clone: 6641897), and Vps25 (pMB 404) into the polycistronic pST39 vector (Tan, 2001) with a 6X-His
tag on the N-terminus of Vps22 (pMB 449). *Xenopus* IMAGE clones were ordered from OpenBiosystems. The coding region of Vps25 was cloned from egg extract. To generate antibodies, the same polycistronic ESCRT-II construct was cloned, except the complex was instead tagged with GST at the N-terminus of Vps36 (pMB 432). Full-length human ESCRT-II and the human ESCRT-II deletion construct lacking membrane binding domains (HuESCRT-II ΔMBD) with 6X-His tags on the N-terminus of Vps22 were gifted from James Hurley (Im and Hurley, 2008). HuESCRT-II ΔMBD is as follows: Vps25(1-176), Vps22(25-258), Vps36(170-386). 6X-His tagged proteins were expressed in BL21 Rosetta, Rosetta(DE3), or Rosetta(DE3)pLysS competent cells. Cultures were grown to an OD 0.4-0.6, then induced with 0.3mM IPTG and grown overnight at 16°C, or were induced at OD 1.0 and grown for 1 hr at 37°C. Cells were collected by centrifugation and resuspended in PBS + 10mM imidazole and protease inhibitors (leupeptin, pepstatin, chymostatin). Cells were lysed by a French press, treated with RNaseA Type IIxa (Sigma) and RQ1 DNase (Promega), then cleared by centrifugation at 12,000 x g for 20 min. Proteins were bound to Ni-NTA agarose (Qiagen), washed with PBS + 10mM imidazole, eluted with PBS + 500mM imidazole, then dialyzed to PBS. Single-use aliquots of purified proteins were snap-frozen in liquid nitrogen and stored at -80°C. GST-ESCRT-II was expressed and induced similarly and was purified with Glutathione Agarose beads (GE Healthcare Life Sciences) according to the manufacturer’s instructions.

**Antibodies and Western blots**

The rabbit polyclonal *Xenopus* ESCRT-II antibody was generated against GST-ESCRT-II by Covance. Serum was affinity purified by incubation overnight at 4°C with 6X-His-ESCRT-II coupled to Affi-Gel 10 agarose beads (Bio-Rad laboratories), and following washes with PBS,
the antibody was eluted from the beads with 0.2M glycine, 500mM NaCl, pH 2.0. The pH of the eluted antibody was neutralized with 1.5M Tris-Cl, pH 8.8 then the eluted antibody was dialyzed to PBS + 50% glycerol. A second batch of *Xenopus* ESCRT-II antibody was generated against 6X-His-ESCRT-II by Covance and purified similarly. Chicken polyclonal antibodies against human ESCRT-II were generated against HuESCRT-II ΔMBD by Aves Labs, Inc. and purified against full length HuESCRT-II. Rabbit polyclonal Trapα antibody was generated against the C-terminal domain (CTD) of *Xenopus* Trapα (residues 230-286) fused to GST and affinity purified against GFP-TrapαCTD as described above. In general, the homemade antibodies were used at 0.2µg/mL for Western and 2µg/mL for immunofluorescence. The following commercial antibodies were used in this study: mouse anti-α-tubulin was used at 1:10,000 for Westerns (DM1α, Sigma), mouse anti-γ-tubulin was used at 1:1000 for immunofluorescence (GTU-88, Sigma), mouse anti-ε-tubulin was used at 1:100 for immunofluorescence (Tub-11, Sigma), rab7 was used at 1:3000 for immunofluorescence (cell signaling, rabbit mAb #9367), and ChromPure Rabbit IgG (whole molecule; Jackson ImmunoResearch). Secondary antibodies for immunofluorescence were used at 1:1000 and were Alexa Fluor® 488 anti-mouse and Alexa Fluor® 546 anti-rabbit (Invitrogen, Molecular Probes) or Cy3 anti-rabbit (Jackson ImmunoResearch). All Western blots were detected with HRP-conjugated secondary antibodies from Jackson ImmunoResearch at 1:10,000. In general, samples were run on a 12% Tris-Glycine SDS-PAGE gel for Western blot.

**Immunofluorescence**

Xl177 cells were fixed in -20°C methanol for 15 min, rehydrated in PBS + 0.1% Triton, blocked in PBS + 0.1% Triton + 1% BSA, then incubated with primary antibodies in block
solution overnight at 4°C. The coverslips were washed 3 times with PBS + 0.1% Triton then incubated with secondary antibodies in block for 1 hr at room temperature. The coverslips were washed 3 times with PBS + 0.1% Triton with DAPI included in the second wash, then mounted in Vectashield® (Vector Laboratories).

Immunofluorescence on sperm nuclei were performed by incubating Xenopus sperm nuclei in egg extract at a concentration of 500nuclei/µl for 15 min in the presence of 10µM nocodazole. Extract was fixed in 4% PFA, 1X BrB80 (80 mM Pipes, pH 6.8, 1 mM MgCl₂, and 1 mM EGTA), 30% glycerol, and 0.5% Triton-X100, then pelleted onto coverslips over a 5mL, 40% glycerol cushion in 1X BrB80 at 10,200 RPM for 10 min at 18°C. Coverslips were fixed in -20°C methanol and immunofluorescence was performed as described above, except with PBS + 0.1% Triton X-100 + 1% milk used as the block.

Spinning disk confocal images were acquired with a 60X 1.42 NA oil objective lens on an Olympus BX61 microscope equipped with a charge-couple device camera (ORCA, Hamamatsu) and were analyzed with the MetaMorph software package (Molecular Devices).

RNA Immunoprecipitations

αESCRT-II or Rabbit IgG was coupled to Protein A Dynabeads (Life Technologies) according to the manufacturer’s instructions. In general, 40µg of antibody and 160µL of Dynabeads were used per 100µL of extract. In some cases, the extract was diluted 1:4 in PBS prior to the immunoprecipitation, and we confirmed that this dilution did not affect the enrichment of candidate RNAs in an ESCRT-II immunoprecipitation. The RNA immunoprecipitations presented in Figure 2.6B were performed from extract diluted in PBS + 0.5% Triton. Extract was incubated with antibody-coupled beads at 4°C for 1-2 hrs. Following incubation, the beads were
washed twice with PBS + 0.5% Triton, twice with PBS, and then the beads were transferred to a fresh tube and resuspended in TRIzol® (Life Technologies). 2µL of the input extract was also resuspended in TRIzol, and RNA was purified from all samples following the manufacturer’s instructions. Purified RNA was resuspended in water and either analyzed on a 0.8% agarose gel with ethidium bromide, used as input for library construction, or used as input for reverse transcription followed by qPCR.

**UV-crosslinking experiments and CLIP-seq**

Native UV-crosslinking experiments in extract were performed by UV crosslinking extract diluted 1:10 in PBS for 10 min on ice in a Stratalinker-2400 (Stratagene) equipped with UVC bulbs. Samples without UV crosslinking were performed in parallel. Samples were treated with 0.1mg/mL RNaseA Type IIx (Sigma) for 30 min at room temperature, then cleared by centrifugation at 10,200 x g for 10 min at 4°C. Samples were pre-cleared with Protein A Dynabeads for 1 hr at 4°C at a ratio of 100µL of beads per 1mL of diluted extract. Samples were then immunoprecipitated with αESCRT-II or mock immunoprecipitated with rabbit IgG and washed as described above for RNA immunoprecipitations. Samples were resuspended in 1X PNK Buffer (NEB) with 1U/µL of RNase Inhibitor (Roche) and 0.75U/µL T4 PNK (NEB) and incubated for 45 min at 37°C to dephosphorylate the 3’ ends of the RNAs. The beads were then washed 3 times in PBS, transferred to a fresh tube, then resuspended in 1X T4 RNA ligase buffer (50mM Tris-HCl, 10mM MgCl₂, 10mM DTT, pH 7.5) with 1mM ATP, 0.02mg/ml BSA, and 1U/µL RNase Inhibitor, and were 3’ end labeled by the addition of 1 µL pCp (cytidine 3’-5’ (bis)phosphate, 5’ 32P, 3000Ci/mmol, PerkinElmer) and 0.5µL T4 RNA ligase (Fermentas) per 20µl reaction. The reactions proceeded for 2 hrs at room temperature, were washed 3 times in
PBS, transferred to a fresh tube, then were resuspended in protein loading buffer, run on SDS-PAGE, transferred to nitrocellulose, and analyzed by autoradiography on a Typhoon Trio scanner (GE Healthcare).

The denaturing UV-crosslinking experiments followed a similar protocol, except the extracts were initially diluted 1:4 in PBS for UV crosslinking, then following the RNAseA treatment, SDS and EDTA were added to a final concentration of 0.5% and 1mM, respectively. The samples were heated at 70°C for 10 min, then diluted 1:2 with PBS + 1% Triton to reduce the SDS concentration for immunoprecipitations.

To construct the CLIP-seq library, UV-crosslinking and denaturing immunoprecipitations were performed as described above except with a low RNaseA concentration (1µg/mL), and instead of 3’ end labeling with pCp, the samples were resuspended in a 20µL mix containing 1X T4 RNA ligase 2, truncated buffer (NEB), 6µl PEG 8000, 1µL RNase inhibitor (Roche), 1µL T4 RNA ligase 2, truncated (NEB), and 30 pmoles of adenylated DNA oligo (oMB1863: 5’ rAppCGGCCGCCACCATCAAT-3ddC 3’). The ligation reaction was incubated overnight at 16°C with agitation in a Thermomixer. The samples were washed, electrophoresed, and transferred as described above, then a region of the nitrocellulose between 31-38kDa was excised and RNA was extracted as previously described (Konig et al., 2011), except with preincubation of the proteinase K solution at 37°C to inactivate RNAses, followed by phenol chloroform extraction. In Figure 2.5A, denatured IPs were 5’ end labeled using the PNK treatment described above except with the addition of [γ-32P]-ATP (PerkinElmer), then the samples were washed as before, resuspended in protein loading buffer, and analyzed by SDS-PAGE and autoradiography as described above. The isolated RNAs were run on a 12% 7.5M urea acrylamide gel and analyzed by autoradiography.
SDS-Page gels used for UV-crosslinking experiments include: 4-12% NuPage Bis-Tris (for native UV CLIP experiment), 12% NuPage Bis-Tris (for low RNase denaturing IPs) run in MOPS running buffer (50mM MOPS, 50mM Tris, 1mM EDTA, 0.1% SDS), or 12% Tris-Glycine (for high RNase denaturing IPs). Westerns of all samples were performed in parallel to monitor immunoprecipitation of ESCRT-II.

CLIP-seq library construction

A custom library protocol was developed using primers and adapters similar to previously described protocols (Ingolia et al., 2012), except with a NotI site inserted into the 3’ adapter and the reverse transcription primer for enzymatic removal of empty adapters without the use of gel purification. The isolated ESCRT-II CLIP RNA was used as input for reverse transcription with 20pg of the oMB1861 primer (5’ (Phos)-CAGATCGGAAGAGCGTGCTGTAGGGAAAGAGTGTAGATCTCGGTGGT CGC-(iSp18)-CACTCA-(iSp18)-TTCAGACGTGTGCTCTTCCGATCTATTGATGGTGTCGCGGCC G 3’) in a 10µL RT reaction using SuperScript III® Reverse Transcriptase (Life Technologies) as described below. The sample was then isopropanol precipitated and resuspended in 1X CircLigase buffer with 50nM ATP, 2.5mM MnCl2 and 0.3µL CircLigase (Epicentre) in a 10µL reaction. The sample was incubated for 1 hr at 60°C, then 10 min at 80°C, then cleaned up with the QIAquick PCR Purification Kit (Qiagen). The sample was PCR amplified using oMB1522 (5’ AATGATACGGCGACCACCGAGATCTACAC 3’) and oMB1904 (5’ TTCAGACGTGTGCTCTTCCG 3’), isopropanol precipitated, then run on an 8% TBE acrylamide gel. The bands containing an insert were gel purified away from unused empty adapters then digested with NotI (NEB) to further remove empty adapters. The sample was
cleaned up with the QIAquick PCR Purification Kit then PCR amplified with oMB1519 (5’ CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCT TCCG 3’) and oMB1522 to add Illumina adapter sequences. After purification with the QIAquick PCR Purification Kit, the sample was submitted for sequencing.

**In vitro UV-crosslinking experiments**

1µg of purified total egg RNA (Figures 2.8B and 2.8D) or 15nM of unlabeled *in vitro* transcribed RNAs (Figure 2.8C) were incubated with the indicated concentrations of ESCRT-II in 100µl of PBS for 30 min at room temperature. The samples were then UV crosslinked for 5 min on ice in a Stratalinker-2400 (Stratagene) equipped with UVC bulbs, then treated with RNaseA (Sigma type XIIA) at 0.1mg/ml for 30 min at room temperature. ESCRT-II was then isolated by immunoprecipitation with αESCRT-II coupled to Protein A Dynabeads, then following washes, the beads were resuspended in PNK buffer and treated with PNK (NEB) and [γ-32P]-ATP (PerkinElmer) for 45 minutes at 37°C. The beads were washed with PBS then resuspended in protein loading buffer and analyzed by SDS-PAGE and autoradiography. In UV-crosslinking experiments including liposomes, brain extract lipids (Folch faction type I, Sigma) were resuspended in a buffer containing 20mM HEPES pH7.4 and 150mM NaCl, then vesicles were formed by sonication followed by two passages through a 0.2µM filter. The filtered solutions were further clarified by centrifugation at maximum speed for 5 min, then the supernatant was added to the *in vitro* binding reactions as described above at the indicated concentrations. All other steps were performed similarly, except the Protein A Dynabeads were additionally washed with PBS + 150mM NaCl + 1% Triton prior to SDS-PAGE.
ESCRT-II human deletion recombinant proteins were UV crosslinked to body-labeled RNA in order to avoid obscuring bands with the PNK-[γ-32P]-ATP intermediate. Body-labeled RNA was in vitro transcribed with T7 RNA polymerase from ctr9 or ctr9 with A’s mutated templates (see RNA EMSA) in the presence of [α-32P]-GTP at a ratio of unlabeled GTP:labeled GTP of 160:1. Following purification by a G25 sephadex (GE Healthcare) column and phenol chloroform extraction, 100μM of ctr9 or ctr9 mutant RNA was used in a 100μL binding reaction with 1μM of the indicated ESCRT-II recombinant protein, and the experiment was performed as described above except without 5’ end labeling and with the anti-HuESCRT-II antibody for the immunoprecipitation. Rabbit anti-chicken (IgY), Fc fragment (Jackson ImmunoResearch) was used to couple the chicken anti-HuESCRT-II to Protein A Dynabeads.

SDS-PAGE was generally performed with 12% Tris-Glycine gels, and in some cases the gels were transferred to nitrocellulose for autoradiography to remove free RNA. Westerns of all experiments were performed in parallel to monitor immunoprecipitation of ESCRT-II.

**RNA isolation from mitotic spindles**

Spindles were prepared in egg extract as described (Hannak and Heald, 2006) and spindle-associated RNAs were isolated as described (Schweidenback et al., 2015). Isolated RNA was either used as input for cDNA libraries, reverse transcription followed by qPCR, or analyzed on 0.8% agarose gels stained with ethidium bromide. To analyze associated proteins, isolated spindles were resuspended in 2X protein loading buffer. To compare spindle-associated RNAs in ESCRT-II- versus mock-depleted extracts, egg extract was incubated with antibody coupled to Protein A Dynabeads for 1 hr at 12°C at a ratio of 100μL extract to 30μg of antibody coupled to
120μL of beads. The beads were removed, and the depleted extract was used to form and isolate spindles.

**Generation of cDNA libraries**

Spindle, RIP-Seq, and RNA-Seq cDNA libraries were prepared using the TrueSeq Stranded mRNA Sample Prep Kit (Illumina). 200ng of all RNA samples for the spindle and RIP-seq experiments were first enriched for mRNA by LNA oligo-dT selection then used as input for library construction. 200ng of total and spindle RNA samples from ESCRT-II- and mock-depleted extracts were enriched for mRNA by selecting for the 5’ mRNA cap (5’ Cap-capture) as previously described (Blower et al., 2013). Additional spindle and RIP-seq cDNA libraries were also prepared using the 5’ Cap-capture approach, and we observed very few differences in the libraries prepared by the two approaches (data not shown). 1μg of all RNA-seq samples from HeLa cells were enriched for mRNA using the Ribo-Zero™ Magnetic Kit (Human/Mouse/Rat) (Epicentre) and used as input for library construction. All libraries were sequenced by SR-50 on the Illumina HiSeq system except the CLIP-seq library, which was sequenced on the Illumina MiSeq system.

**RT-PCR and Quantitative PCR**

In general, 200ng of RNA samples were used for reverse transcription with random hexamers and SuperScript III® Reverse Transcriptase (Life Technologies) according to the manufacturer’s instructions. Samples were treated with RNAselH (NEB) for 20 min at 37°C following the reverse transcription reaction. cDNAs were used as input for qPCR with iQ™
SYBR®Green supermix (Bio-Rad Laboratories) and primer sets listed in Appendix I, Table S.1. qPCRs for each biological replicate were performed in triplicate.

To analyze splice variants, reverse transcription was performed as described above, then the cDNA was used as the template for PCR with primers flanking the predicted skipped exon (see Appendix I, Table S.1). Samples were analyzed on a 7% TBE acrylamide gel, stained with SYBR® Gold (Life Technologies), and visualized on a Typhoon scanner.

RNA EMSA

RNA probes for electrophoretic mobility shift assays (EMSA) were generated by annealing complementary phosphorylated oligos of the sequences indicated in Figures 2.9-2.11 with overhanging NotI and BamHI overhanging sequences to clone into the pCR2.1 vector. PCR products were generated using M13 forward (5’ GAAAACGACCGCCAG 3’) and reverse ( 5’ CAGGAAACAGCTATGACC 3’) primers and were used as templates for in vitro transcription with T7 RNA polymerase. Therefore, all EMSA probes were 40nts of unique sequences plus identical flanking sequences of the pCR2.1 vector between the T7 to NotI sites and the BamH1 to M13 sites. In vitro transcription reactions were supplemented with Cy5-UTP (GE Healthcare) at a ratio of unlabeled:labeled UTP of 2:1 or with [α-32P]-ATP at a ratio of unlabeled:labeled ATP of 160:1. We found that ESCRT-II binds similarly (although with a slightly lower affinity) to fluorescently labeled probes compared to radioactively labeled RNA probes. After completion, in vitro transcription reactions were DNase treated, then purified by LiCl precipitation and resuspended in water. 1nM of fluorescently-labeled RNA probe or 200pM of radioactively-labeled RNA probe was used for EMSA. RNA probes were first heated to 65°C for 5 min then cooled to room temperature to remove secondary structure. Gel shifts were performed
in 1X RNA binding buffer (10mM HEPES, 100mM KCl, 1mM MgCl₂, 1mM EDTA, 5% Glycerol, 1mM DTT, pH 7.7) in the presence of yeast tRNA at 0.1mg/mL and 0.8U/µL of RNase inhibitor (Roche). HuESCRT-II ∆MBD was used for gel shifts at the indicated concentrations. Binding reactions were allowed to proceed for 30 minutes at 20°C, then were loaded onto a native 8% TBE acrylamide gel (with 19:1 acrylamide:bis) that was pre-run for 30 min at 150V. Gels were run at 150V for 1 hr in 0.5X TBE then analyzed by autoradiography or for fluorescence on a Typhoon scanner. Bands were quantified using ImageQuant (Amersham) and curves were fit to the Hill equation using R, allowing the hill coefficient to float.

**Cell culture, siRNA, and RNA isolation from cultured cells**

*Xenopus* XI177 cells were cultured in Leibovitz's L-15 media (Sigma) with 10% FBS. esiRNAs were generated and purified as previously described (Sharp et al., 2011) against regions of the *Xenopus* Vps25 or GFP transcripts, as defined by the PCR primers listed in Appendix I, Table S.1 that were used to generate the templates. 100pmoles of esiRNAs and 1µg *Xenopus tropocalis* Ago2 plasmid (pMB 886) were transfected into 3 x 10⁶ cells using an Amaxa Nucleofector II, solution V, and program T-020 (Lonza), according to the manufacturer’s instructions. Cells were grown for three days then were harvested in 2X protein loading buffer and analyzed by Western blot, or in Buffer RLT + 1% BME of the RNeasy kit (Qiagen) and processed according to the manufacturer’s instructions. RNA samples were analyzed by qPCR to measure knockdown of Vps25 RNA.

HeLa cells were cultured in DMEM + 10% FBS. esiRNAs were designed against the 3’ UTR of Human Vps25 or against a region of Human Vps28 as defined by the PCR primers listed in Appendix I, Table S.1. Additionally, a commercial siRNA against HuVps25 was obtained.
from Ambion (silencer siRNA #34229). 10pmols of HuVps25 esiRNA or GFP esiRNA or 5pmols of the commercial HuVps25 siRNA or a negative control siRNA (negative control #1, AM4611, Ambion) were transfected into HeLa cells using Lipofectamine® 2000 (Life Technologies) or Lipofectamine® RNAiMax (Life Technologies). The cells were cultured for 4 days then were harvested for Western blot and RNA as described above. The isolated RNA was used for qPCR as described above, used as input for RNA-seq, or used as input for RT-PCR to validate differential splicing.

**Analysis if Xenopus cDNA libraries**

Sequencing reads from the Illumina Hi-Seq were collapsed into unique reads using a custom Perl script (Schwarz and Blower, 2014). Reads aligning to *Xenopus laevis* rRNA sequences were subtracted using a custom Perl script then were aligned to a draft version of the *X. laevis* genome (version 7.0, downloaded from Xenbase.org) using TopHat and Cufflinks (Trapnell et al., 2010). Reads were counted against a custom gene model (Schweidenback et al., 2015) using the cuffdiff program. Only transcripts with more than 100 combined normalized alignment counts were included in further analyses.

To perform Gene Ontology (GO), we used BlastX to search the *X. laevis* transcripts generated by Cufflinks to the Human Uniprot database and retained all hits with an e-value of \(< 1 \times 10^{-20}\). We then used the Human Uniprot accession numbers as input for GO enrichment analysis using NCBI DAVID (Huang da et al., 2009a, b). For background in this analysis, we used all expressed transcripts in which all replicates of the experiment had greater than 100 combined reads.
Analysis of HeLa cell cDNA libraries

To compare the abundance of transcripts under different siRNA conditions, unique reads from the RNA-seq libraries were aligned to the Human Refseq mRNA database using Bowtie (Langmead et al., 2009), allowing up to 2 mismatches and up to 5 alignments. Reads per transcript were calculated using a custom Perl script, and transcripts with less than 100 combined reads were removed for further analysis. To analyze differential splicing, unique reads were aligned to the human genome sequence (Hg19) using TopHat then were analyzed using MATs (Park et al., 2013).

Analysis of CLIP-seq cDNA library

FastQ sequences were filtered to remove linker sequences then were collapsed into unique reads using a custom Perl script. Reads with >90% of a single base were removed using a custom Perl script. The remaining reads were aligned to X. laevis rRNA genes using Bowtie allowing for up to 3 mismatches, and the aligning reads were removed. The remaining reads were aligned to sequences derived from a custom gene model (Schweidenback et al., 2015) allowing up to 3 mismatches and retaining the best strata of alignments using the parameters –m 3 –best –strata. Reads aligning to more than two transcripts or to antisense strands were removed. Reads of equal length aligning to the same position of the transcriptome were removed under the assumption that these reads result from overamplification and mutations acquired during the PCR steps of library preparation. The alignments were counted with a weighted counting strategy using a custom Perl script, such that if a read aligned twice, each transcript received a count of 0.5. We used the samtools mpileup command to create coverage files, and a custom Perl script was used to analyze transcripts with multiple aligning reads. To perform motif analysis, we collapsed overlapping reads into CLIP-tags and extended the sequences with 25 nts
of flanking sequence on either side of the CLIP-tag. The resulting sequences were used as input for motif prediction using MEME (Bailey et al., 2009).

**Analysis of dinucleotide or mononucleotide motifs in the transcriptome**

We used a 30 nt sliding window to identify windows with the highest percentage of all possible dinucleotide or mononucleotide combinations. To identify transcripts with dinucleotide or mononucleotide motifs, we selected transcripts containing the top 2-3 mismatch bins (e.g. for AG we allowed up to 3 non-AG nucleotides in a 30 nt window). These subsets of transcripts were used to compare enrichment in ESCRT-II immunoprecipitations to the total RIP-seq library. Statistical tests to compare these distributions were performed using a two-sided t-test in R.

**Analysis of relative location of ESCRT-II CLIP-tags and motifs**

To identify the locations of start and stop codons, we used BlastX to map the alignment of JGI annotated protein sequences (xenbase.org) to our custom transcriptome. We then used the first nucleotide of each ESCRT-II CLIP-tag (from the pileup coverage files of ESCRT-II CLIP sequences) or the identified dinucleotide motifs (above) to determine if the sequences are present in the 5’UTR, coding region, or 3’UTR. For each sequence feature (e.g. 5’UTR), we divided the feature into 10 bins of equal length and created a histogram of the normalized distribution of the CLIP-tags or motifs within each feature. To determine the percentage of CLIP-tags or motifs at each normalized position, we created a vector containing the counts of CLIP-tags or motifs in the 5’UTR, coding region, and 3’UTR (now 30 bins long) and divided each bin of this vector by the total number of CLIP-tags or the relevant motifs. Graphs were prepared using R.
**Appendix I: Supplemental Tables**

**Table S.1: Primers used in this dissertation.** All genes are from *Xenopus laevis* unless otherwise indicated.

<table>
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<th>Gene</th>
<th>Forward primer</th>
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<td>AAGCGGTTGGAATAAAAACCTTG</td>
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<tr>
<td>Human vps25</td>
<td>AGGGAATCTCGGTCTTGTGGGGG</td>
<td>ATCAATCAGTTCAGTTCAGAAGT</td>
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<td>Human vps28</td>
<td>ACAAGCTGCAAGCGCTAGGGG</td>
<td>AGAGAAGTCTGAGCCTCCGG</td>
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<tr>
<td>GFP</td>
<td>GGCGGGGTTACCGCGAGAGCTGTTCA</td>
<td>GGCGGGGTTACCGCGAGAGCTGTTCA</td>
</tr>
<tr>
<td>GFP 2nd PCR</td>
<td>TAATACGACTCACTATAGGGGACGACGCAC</td>
<td>GGCGGGGTTACCGCGAGAGCTGTTCA</td>
</tr>
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</table>

All sequences except for GFP primers start with "ACATGATAATACGACTCACTAGG" to add the T7 promoter. GFP templates underwent a second PCR with an additional primer to add the T7 promoter.
References


