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Dis3- and exosome subunit-responsive 3′ mRNA instability elements

Daniel L. Kiss [a,1,2], Dezhi Hou [a,1], Robert H. Gross [b], Erik D. Andrulis [a,*]

[a] Case Western Reserve University School of Medicine, Department of Molecular Biology and Microbiology, Cleveland, OH 44106, United States
[b] Dartmouth College, Department of Biological Sciences, Life Sciences Center 343, Hanover, NH 03755, United States

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ABSTRACT

Eukaryotic RNA turnover is regulated in part by the exosome, a nuclear and cytoplasmic complex of ribonucleases (RNases) and RNA-binding proteins. The major RNase of the complex is thought to be Dis3, a multi-functional 3′–5′ exoribonuclease and endoribonuclease. Although it is known that Dis3 and core exosome subunits are recruited to transcriptionally active genes and to messenger RNA (mRNA) substrates, this recruitment is thought to occur indirectly. We sought to discover *cis*-acting elements that recruit Dis3 or other exosome subunits. Using a bioinformatic tool called RNA SCOPE to screen the 3′ untranslated regions of up-regulated transcripts from our published Dis3 depletion-derived transcriptomic data set, we identified several motifs as candidate instability elements. Secondary screening using a luciferase reporter system revealed that one cassette—harboring four elements—destabilized the reporter transcript. RNAi-based depletion of Dis3, Rrp6, Rrp4, Rrp40, or Rrp46 diminished the efficacy of cassette-mediated destabilization. Truncation analysis of the cassette showed that two exosome subunit-sensitive elements (ESSEs) destabilized the reporter. Point-directed mutagenesis of ESSE abrogated the destabilization effect. An examination of the transcriptomic data from exosome subunit depletion-based microarrays revealed that mRNAs with ESSEs are found in every up-regulated mRNA data set but are underrepresented or missing from the down-regulated data sets. Taken together, our findings imply a potentially novel mechanism of mRNA turnover that involves direct Dis3 and other exosome subunit recruitment to and/or regulation on mRNA substrates.

1. Introduction

Messenger RNA (mRNA) turnover is essential for maintaining proper gene expression and cell function. Surplus or aberrant mRNAs—either mis-transcribed, mis-processed, mis-transported, mis-folded, mis-packaged, mis-modified, or mutated—must be identified, sequestered, and degraded in a precise manner. These phenomena, included under the umbrella term mRNA surveillance [1,2], require the functional and physical interplay of a network of mRNA metabolic processes. Although much progress has been made towards understanding many of the pathways that comprise the network [3–5], the molecular mechanisms by which aberrant or surplus mRNAs are detected and winnowed are still unclear.

The exosome complex and its cofactors have emerged over the last decade as fundamental participants in mRNA surveillance. Structurally, the yeast exosome core is composed of a hexameric ring consisting of RNase PH subunits (Rrp41/Ski6, Rrp42, Rrp43, Rrp45, Rrp46, and Mtr3) and a trimeric cap consisting of S1 domain subunits (Rrp4, Rrp40, and Csl4) [6]. Two active ribonucleases interact with the core: Rrp6, an RNase D homolog, and Dis3, an RNase II/R homolog. Functionally, these exosome subunits and RNases have been implicated in most if not every aspect of mRNA metabolism including elongation [7,8], splicing [9–11], 3′ processing [12–14], termination [15,16], transport [17,18], and turnover [19,20]. While there is evidence suggesting that the entire core complex participates in mRNA surveillance, there is also evidence indicating that individual subunits "survey" mRNAs independently of the complex [21–23]—either alone or as subcomplexes that we have called exozymes [24]. One recent study that supports the exozyme hypothesis showed that patients with pontocerebellar hypoplasia type 1 have specific, non-lethal mutations in EXOSC3 (Rrp40) [25]. These mutations have tissue-specific effects, suggesting that Rrp40 regulates a subset of exosome core-surveyed RNAs.

Abbreviations: SCOPE, suite for computational identification of promoter elements; ESSE, exosome subunit-sensitive element; RNase, ribonuclease.

* Corresponding author. Address: Case Western Reserve University School of Medicine, Department of Molecular Biology and Microbiology, 10900 Euclid Avenue, Wood Building, W212, Cleveland, OH 44106, United States. Fax: +1 216 368 3055.
E-mail address: exa32@case.edu (E.D. Andrulis).

[1] These authors contributed equally to this work.

[2] Present address: Center for RNA Biology, Ohio State University, School of Biomedical Sciences, 385 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210, United States.

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Dis3 and Rrp6 have attracted a great deal of attention because they exist in a biochemically defined exozyme [26,27], have both exosome-tethered and -independent ribonucleolytic activity [6,21–23,27–31] and modulate cell cycle progression [32–34]. In light of this functional multivalence, making the biochemical distinction between the core complex and exozymes is necessary for our understanding of how these essential polypeptides recognize and process or degrade distinct RNA substrates.

A critical step in 3′–5′ mRNA decay is protein recruitment to the 3′ untranslated region (UTR) of the mRNA substrate [35]. Several non-exosome RNA binding proteins have been suggested or shown to elicit AU-rich element (ARE)-mediated mRNA turnover through recruitment of the exosome or subunits of the complex [36–38]. Several RNase PH subunits preferentially bind AREs, triggering their turnover [39,40]. Still, we do not have a complete understanding of which exosome subunits and exozymes directly recognize specific RNA sequence motifs or structures. In this regard, most if not all exosome subunits have bioinformatically predicted or experimentally defined specific RNA interaction domains [41–44], some of which are known to bind particular RNA structures [45,46]. Moreover, several models have suggested or implied a direct RNA-S1 cap interaction prior to RNA threading through the hexameric RNase PH ring and ultimate catalysis by Dis3 [47,48].

Here, we use bioinformatics to identify novel 3′ UTR instability elements. We characterize two elements that confer Dis3- and exosome subunit-sensitive instability in a reporter system. These *cis*-acting elements exist almost exclusively within the transcriptomic pool that is stabilized by depleting exosome subunits, suggesting that they are targets for exozyme or exosome recruitment and Dis3-mediated decay.

2. Materials and methods

2.1. Antibodies and S2 cell culture

Polyclonal exosome subunit-specific primary antibodies and HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and *Drosophila melanogaster* S2 cell culture were described earlier [7,34].

2.2. Double-stranded RNA preparation and RNA interference

Double-stranded RNA (dsRNA) was prepared as previously described [20,34]. Cells were treated with dsRNAs (30 μg/mL) on days 0, 1, and 3 and harvested on the 5th day [34].

2.3. Luciferase reporter assays

Cis-elements were added to luciferase reporters (Addgene) and using the Change-IT kit (USB). S2 cells were co-transfected either with empty pAC5.1c-Fluc or with pAC5.1c containing *cis*-element as indicated and with pAC5.1c-Rluc using Cellfectin II (Invitrogen). Cells were lysed using the PLB buffer from dual luciferase reporter assay system (Promega) the day after transfection. Firefly and renilla luciferase levels were determined in triplicate.

2.4. Real-time PCR

RNA was isolated from S2 cells with TRIzol Reagent (Invitrogen), cleaned with RNAeasy mini kit (Qiagen), and first-strand cDNA synthesis performed (on 1 μg RNA) with the Quantitect Reverse Transcription kit (Qiagen). Real-time PCR was performed with SYBR Green PCR master mix (Qiagen) and BIO-RAD iCycler IQ real-time PCR system. Primers (Table S1) were custom designed (Primer3).

3. Results

3.1. Bioinformatic identification of candidate cis-acting instability elements

We selected our previously published Dis3 microarray data for bioinformatic screening of *cis*-acting instability elements because our ongoing interest in characterizing Dis3 [20,27,29,33]. RNA motif identification was performed using a modification of the SCOPE program [49] designed to work on RNA regions instead of DNA (RNA SCOPE [50]). Using RNA SCOPE, we identified 84 candidate conserved sequence elements. We reasoned that regulatory elements would be rare, so we used a 1 in ~4000 chance (*i.e.*, a completely conserved 6-mer motif) as a lower boundary for these elements. At that scarcity, such an element would occur by chance in roughly one tenth of *Drosophila* 3′ UTRs (average length of ~375 nt) [20]. After excluding motifs that were underrepresented in the transcriptome, had weak consensus sequences, or could not be tested conclusively (Fig. 1), 17 RNA SCOPE-defined motifs remained.

3.2. Experimental screening of candidate instability elements

We engineered four sets of contiguous candidate elements—with linker nucleotides between each element—into independent cassettes. These cassettes (M1, M2, M3, and M4) were cloned into the 3′ UTR of a firefly luciferase reporter plasmid, and screened by a luminescence-based assay in transiently transfected *Drosophila* S2 tissue culture cells (Fig. 2a). A comparison of these cassettes to the empty vector showed that M2 had no effect, M1 and M3 showed a 10–20% reduction in signal, and the M4 cassette elicited >90% reduction in signal (Fig. 2b).

Given that the luciferase assay measures luminescence (protein levels), we test whether the M4-dependent reduction of light signal was caused by reduced RNA level. To this end, we performed quantitative real-time RT-PCR on total RNA purified from S2 cells transfected with either the control (empty) vector or the

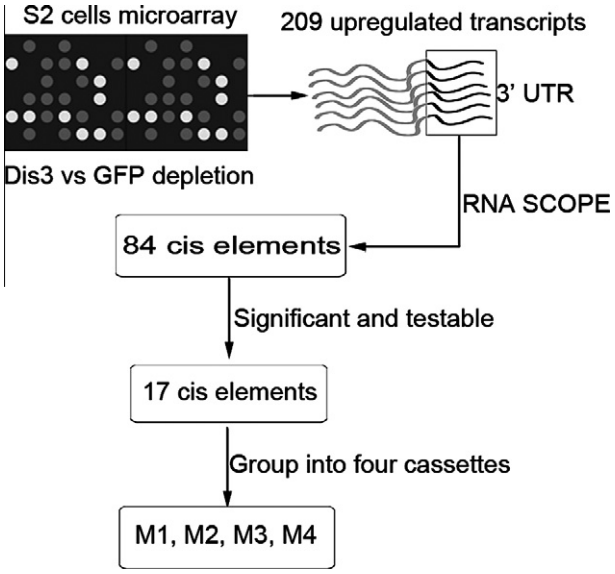

Fig. 1. Multi-step screen to identify candidate Dis3-responsive 3′ UTR instability elements. RNA SCOPE used Flymine to fetch 3′ UTR sequences from the transcript IDs; these transcripts were specifically stabilized in the Dis3 depletion-based microarray.

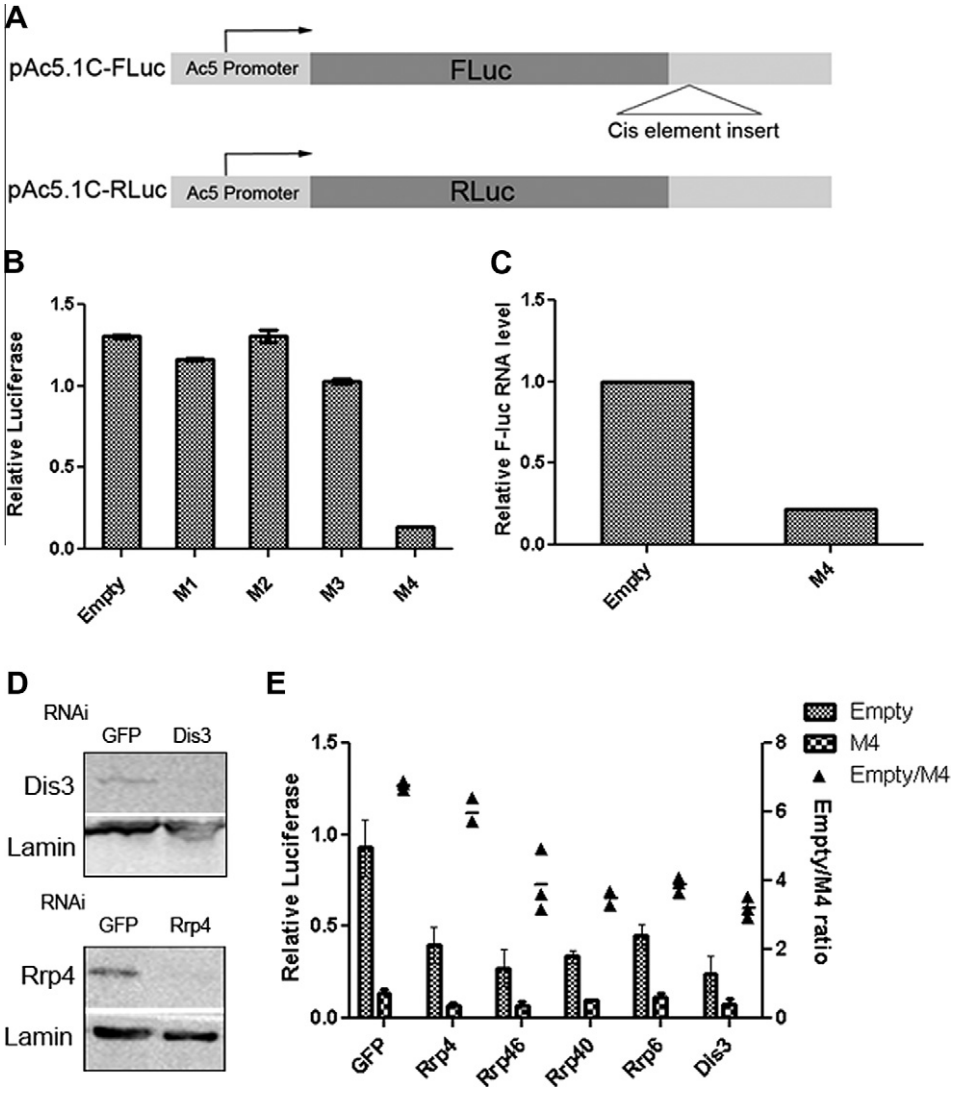

Fig. 2. Identification and characterization of novel mRNA instability element. (A) Constructs used in dual luciferase assay: Fluc, firefly luciferase; Rluc, renilla luciferase. Candidate *cis*-elements inserted into 3′ UTR of firefly luciferase. (B) Testing four multiple elements (M1, M2, M3, and M4) for destabilizing effect. Relative luciferase refers to firefly luciferase normalized to renilla luciferase (internal control). (C) Real-time RT-PCR reveals that the M4 insert destabilizes the firefly luciferase mRNA. (D) Exosome subunit Rrp4 and Dis3 are effectively depleted by RNAi in S2 cells. Lamin is the loading control. (E) M4 requires proper exosome subunit levels to destabilize firefly luciferase. Note that RNAi depletion of Dis3 elicits the greatest effect.

M4-containing vector. The 4-fold decrease in relative mRNA levels (Fig. 2c) indicates that M4 destabilizes the reporter mRNA.

Since these elements were identified in mRNAs up-regulated by Dis3 depletion, we expect M4-dependent destabilization of firefly luciferase mRNA to require Dis3. Using RNAi to deplete Dis3 or GFP (Fig. 2d), we captured the raw data in triplicate for either the empty or M4-containing reporter (Fig. 2e). Because Dis3 depletion affects the levels of the empty reporter, we compared the ratio of empty to M4 (Empty/M4) for each experimental sample. By this analysis, Dis3 depletion has a greater than 2-fold reduction in this ratio as compared to GFP depletion alone. To determine whether the M4-mediated destabilization of the reporter required other exosome subunits, we depleted Rrp6, Rrp4, Rrp40, and Rrp46 using RNAi (Fig. 2d). For each subunit, the relative Empty/M4 ratio was statistically lower than that of GFP (Fig. 2e). Together, these data suggest that Dis3, Rrp6 and core exosome subunits are each necessary for effective M4-mediated destabilization of the firefly luciferase mRNA.

3.3. M4 cassette deletion and mutagenesis defines two sequence motifs

To identify which of the four element(s) in M4 w(as/ere) responsible for the destabilization effect, we tested a set of truncations (Fig. 3a). We found modest effects—30–40% reduction—when the four elements (named A, B, C, or D) were tested independently. These modest effects are surprising because RNA SCOPE identified unique, *individual* sequences and because they are not consistent with the robust M4-mediated effects (Fig. 2). Thus, we tested the elements in pairs to make sense of this anomaly. Although we did not observe additive or synergistic effects with either the AB or BC element pairs, we saw a striking and specific effect with the CD element pair. We henceforth refer to these two elements together as the exosome subunit-sensitive elements (ESSEs), with C and D being called ESSE1 and ESSE2, respectively. We pursued a deeper understanding of ESSE-dependent destabilization through site-directed mutagenesis. Single and triple point mutations in either ESSE1 or ESSE2 alone or both together restored the

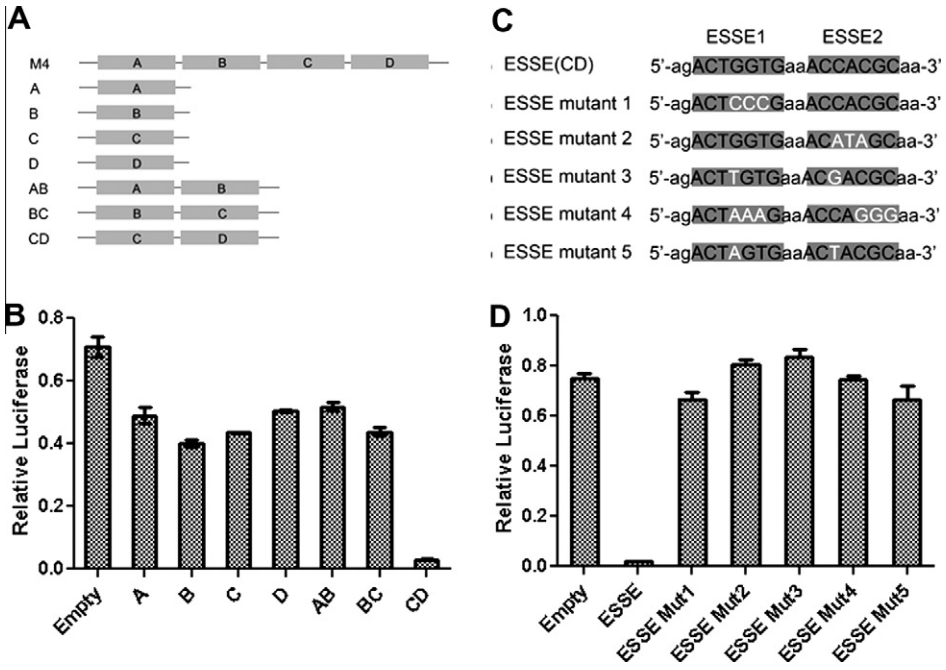

Fig. 3. ESSE: the exosome subunit-sensitive element in M4. (A) Schematic of truncation series of the four candidate *cis*-acting instability elements in M4. (B) Testing the truncations in the luciferase reporter assay reveals that CD confers instability; henceforth, CD is referred to as ESSE, for exosome subunit-sensitive element. (C) Schema of point mutation on ESSE elements, with ESSE1 and ESSE2 as noted. (D) Luciferase reporter assay reveals that both ESSE1 and ESSE2 are required for ESSE-mediated instability.

Table 1
Number of ESSE-containing transcripts affected by exosome subunit depletion.

	ESSE1		ESSE2		ESSE1 & 2	
Depletion	Up	Dn	Up	Dn	Up	Dn
Dis3	8	3	11	3	2	0
Rrp6	1	2	1	0	0	0
Rrp47	15	1	16	0	2	0
Mtr3	34	0	27	0	6	0
Ski6	33	1	29	0	7	0
Rrp46	9	1	11	2	7	0
Rrp4	27	2	24	0	3	0
Rrp40	12	0	11	2	3	0
Csl4	9	2	8	1	2	0

luciferase activity that was abrogated by the wild-type ESSE (Fig. 3c and d).

3.4. ESSE is enriched in exosome subunit-depleted, stabilized transcriptomes

We next examined the scope and frequency of ESSE in our complete published microarray data set [20]. In quantifying the number of transcripts that contained either ESSE1 or ESSE2 alone or both together (Table 1), we found that RNase PH subunits (Mtr3, 61; Ski6, 62; Rrp46, 20) had the most ESSE-containing transcripts, S1 subunits had the next greatest (Rrp4, 51; Rrp40, 23; Csl4, 17), and the core exosome-associated proteins had the fewest (Dis3, 19; Rrp6, 2; Rrp47, 31). A similar frequency was found for both ESSE1 and ESSE2 in an individual stabilized transcript. In contrast to the stabilized RNAs, ESSE1 and ESSE2 were underrepresented in the down-regulated transcripts (Table 1, bottom). There were no down-regulated mRNAs containing both ESSE1 and ESSE2. These data support the idea that the bipartite ESSE is a *bona fide* instability element.

Analyzing the 89 ESSE1- and ESSE2-containing transcripts stabilized by exosome subunit depletion, we quantified how many

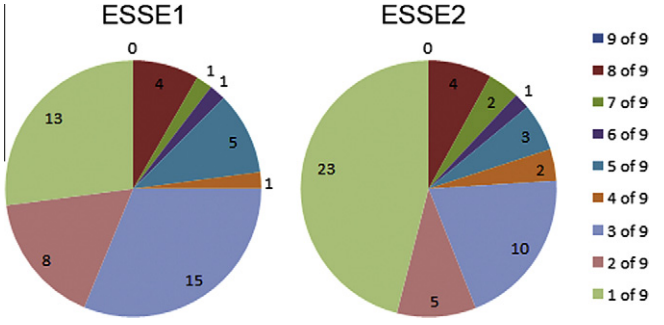

Fig. 4. Shared up-regulated transcripts with ESSE across multiple depleted exosome subunit microarrays. Frequency that ESSE1 (left pie chart)- and ESSE2 (right)-containing transcripts are co-regulated by one or more exosome subunits.

of these are affected by the full complement of exosome subunits (Fig. 4). We find that there was not one ESSE-containing RNA that was affected by the depletion of all subunits tested. By comparison, about 25% of the transcripts are stabilized by the depletion of 4 or more exosome subunits.

4. Discussion

We use a computational analysis to identify novel *cis*-acting mRNA targets of exosome subunits. Through motif isolation, mutagenesis, and RNAi-based testing in a reporter assay system, we discovered the ESSE: a pair of *cis*-acting elements that confer mRNA destabilization. ESSE is enriched in the stabilized mRNA pools for all exosome subunit depletion microarray data sets.

4.1. Mechanisms and compartments of exosome-mediated RNA decay

To the best of our knowledge, this work is the first to identify specific *cis*-acting 3′ UTR mRNA sequence elements to which the exosome, exozyme, or set thereof may be recruited. Whether this

recruitment occurs in the nucleus—either co- or post-transcriptionally—or cytoplasm is undetermined but may occur in either one or both compartments [51]. Additional experiments are necessary to clarify which, if any, subunit(s) bind(s) directly to ESSE—or are recruited indirectly by an undefined RNA binding protein—and destabilize(s) ESSE-containing mRNAs. Given the observed difference of the 4-fold and 10-fold decrease in luciferase mRNA and protein, respectively, we suspect that the ESSE elicits some of its effects at the level of translation.

4.2. Is the cis-acting element reconstituting a trans-acting phenomenon?

The observation that ESSE-mediated destabilization required both ESSE1 and ESSE2 was quite surprising because RNA SCOPE was used to identify individual elements. We propose three interpretations to make sense of this unusual outcome. First, discovery of ESSE is an artifact of the cloning process or placement in the reporter; its activity does not reflect a ribo-metabolically relevant phenomenon. However, both ESSE1 and ESSE2 individually confer a ~40% reduction in mRNA stability. Further, the ESSE-dependent destabilization effect is sensitive to the protein levels Dis3, Rrp6, and core exosome subunits. Moreover, ESSE1 and ESSE2 are enriched *only* in stabilized transcript sets. Thus, though we cannot rule out this possibility, it is unlikely. A second possibility is that RNA SCOPE identified ESSE1 and ESSE2 together in a limited number of stabilized transcripts—even though they also exist in independent transcripts. Then, of the enriched elements selected for further analysis, ESSE1 and ESSE2 were by coincidence subcloned together. While this scenario is possible, it seems unreasonable. We considered a third possibility: the ESSE, a single *cis*-acting RNA unit of two apposed elements, reconstitutes a previously undiscovered *trans*-acting phenomenon. That is, under normal conditions in wild-type cells, ESSE1 resides in the 3′ UTR of one mRNA and ESSE2 is in the 3′ UTR of another, non-contiguous mRNA. In this hypothetical scenario, the exosome or an exozyme acts as a molecular bridge between the two elements, coupling two mRNAs together and targeting them simultaneously for degradation. While this protein bridging is thought to occur within RNA molecules [52] (as within and between DNA [53,54]), this mechanism of action requires experimental validation.

4.3. Can we use ESSE frequency to test the exozyme hypothesis?

The exozyme hypothesis [24] predicts that most ESSE-containing transcripts should not overlap between the microarray profiles of depleted exosome subunits. Consistent with this prediction, ~50% of ESSE1 or ESSE2-containing mRNAs are shared in only two exosome-subunit depletion microarray data sets (mostly Mtr3 and Ski6). That a varying number of subunits act on ESSE ultimately suggests that there is great deal of cross-talk between exosome subunits and factors in 5′–3′ decay and 3′ mRNA processing. Thus, while our data provides support for the exozyme hypothesis, it does not rule out the possibility that, for example, Mtr3 and Ski6 impact ESSE-containing mRNAs in the context of the exosome core.

In summary, we have identified two potential Dis3- and exosome-directed instability elements in 3′ UTRs of certain *Drosophila* mRNAs using RNA SCOPE. Based upon ESSE enrichment in our microarray-identified stabilized RNA pool, we suggest that the exosome and/or exozymes may be directly recruited to these mRNAs. Given the widespread roles for Dis3 and the exosome in mRNA turnover, processing, transport, packaging, and surveillance, our study provides new insight into how they regulate distinct steps in general mRNA metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.141.

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