The Transcriptome-wide Landscape and Modalities of EJC Binding in Adult *Drosophila*

**Graphical Abstract**

**Highlights**
- ipaRt: a method for protein-RNA-binding landscape definition in cells and organisms
- *Drosophila* EJC-bound mRNAs are biased toward differentiation and development
- *Drosophila* EJC assembly on mRNAs favors genes with complex gene architecture
- Splice site strength, RNA structure, and CG-rich hexamers enhance EJC binding in flies

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**In Brief**
Obrdlik et al. present ipaRt, an approach for definition of the EJC-RNA-binding landscape in adult *Drosophila melanogaster*. Their study uncovers the impact of gene architecture, splice site strength, RNA structures, and CUG hexamers on EJC binding and provides insights into the evolution of EJC functions.
The Transcriptome-wide Landscape and Modalities of EJC Binding in Adult Drosophila

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SUMMARY

Exon junction complex (EJC) assembles after splicing at specific positions upstream of exon-exon junctions in mRNAs of all higher eukaryotes, affecting major regulatory events. In mammalian cell cytoplasm, EJC is essential for efficient RNA surveillance, while in Drosophila, EJC is essential for localization of oskar mRNA. Here we developed a method for isolation of protein complexes and associated RNA targets (ipaRt) to explore the EJC RNA-binding landscape in a transcriptome-wide manner in adult Drosophila. We find the EJC at canonical positions, preferably on mRNAs from genes comprising multiple splice sites and long introns. Moreover, EJC occupancy is highest at junctions adjacent to strong splice sites, CG-rich hexamers, and RNA structures. Highly occupied mRNAs tend to be maternally localized and derive from genes involved in differentiation or development. These modalities, which have not been reported in mammals, specify EJC assembly on a biologically coherent set of transcripts in Drosophila.

INTRODUCTION

The exon junction complex (EJC) consists of a heterotetramer core composed of elf4AII, Mago, Y14, and Barentsz (Btz) (Bono et al., 2006; Stroupe et al., 2006) and auxiliary factors that form the EJC periphery (Tange et al., 2005). The complex assembles on mRNAs during splicing, ~20 to ~24 nt upstream of exon-exon junctions (Le Hir et al., 2000). EJC assembly is a multi-step process that begins with CWC22-mediated deposition of the DEAD-box helicase elf4AII on nascent pre-mRNAs (Alexandrov et al., 2012; Barbosa et al., 2012; Steckelberg et al., 2015) and is followed by recruitment of Mago and Y14, forming a pre-EJC intermediate. The pre-EJC is stably bound to RNA because of the ATPase-inhibiting activity of the (non-RNA-binding) Mago–Y14 heterodimer, which “locks” elf4AII helicase in its RNA-bound state (Andersen et al., 2006; Ballut et al., 2005; Bono et al., 2006; Stroupe et al., 2006). Once formed, the pre-EJC is completed by recruitment of Barentsz (Btz), forming mature EJCs (Bono et al., 2006; Bono and Gehring, 2011; Tange et al., 2005). The roles of the EJC in post-transcriptional control of gene expression are manifold. In the nucleus, EJC subunits have a role in splicing (Ashton-Beaucage and Therrien, 2011; Ashton-Beaucage et al., 2010; Roignant and Treisman, 2010), mRNA export (Gatfield et al., 2001), and nuclear retention of intron-containing RNAs (Shimori et al., 2013). In the cytoplasm, the EJC is reported to play a role in translation (Chazal et al., 2013; Nott et al., 2004), nonsense-mediated decay (NMD) (Buchwald et al., 2010; Gehring et al., 2005; Melero et al., 2012; Okada-Katsuhata et al., 2012; Palacios et al., 2004; Shibuya et al., 2006; Singh et al., 2007), and RNA localization (Ghosh et al., 2012; Hachet and Ephrussi, 2001, 2004; Palacios et al., 2004; van Eden et al., 2001). Although most EJC functions appear conserved, in Drosophila the EJC is not crucial for NMD (Behm-Ansmant et al., 2007), but it is essential for oskar mRNA localization within the developing oocyte (Ghosh et al., 2012, 2014; Hachet and Ephrussi, 2001, 2004; Palacios et al., 2004; van Eden et al., 2001; Zimyanin et al., 2008). To better understand the engagement of the EJC in the fly, we developed a strategy to stabilize mRNA binding proteins (mRBPs) associated with their RNA templates within multi-protein messenger ribonucleoprotein (mRNP) assemblies and set out to define the EJC mRNA interactome in adult Drosophila melanogaster. Through the use of the crosslinking agent dithio(bis-)-succinimidylpropio-nate (DSP), our method captures stable and transient protein interactions in close proximity (Lomant and Fairbanks, 1976; Schweizer et al., 1982) and allows definition of the binding sites of specific protein (holo-)complexes associated with their RNA templates (isolation of protein complexes and associated RNA targets [ipaRt]). Our analysis of EJC-protected sites defined by ipaRt reveals that in Drosophila, EJC binding occurs at canonical deposition sites (Le Hir et al., 2000), with a median coordinate ~22 nt upstream of exon-exon junctions. Although in mammals EJC-mediated protection outside canonical sides was reported (Saulière et al., 2012; Singh et al., 2012), we find that in Drosophila the degree of non-canonical EJC-mediated RNA protection is minimal. We show in Drosophila that RNA polymerase II transcripts protected primarily by the EJC derive from genes involved in differentiation or development, while mRNAs protected primarily by mRBPs derive from genes with homeostatic functions. Our analysis suggests that the EJC’s bias for transcripts in Drosophila is a consequence of several modalities in the genes’ architecture, particularly splice site number and intron
length. Moreover, EJC binding is enhanced by adjacent RNA secondary structures and CUG-rich hexamers located 3’ to the EJC binding site. These modalities were not identified in previous studies of mammalian EJC binding (Hauser et al., 2016; Saulière et al., 2012; Singh et al., 2012), reflecting either greater specificity of our method for fully assembled EJCs or differences in EJC binding between flies and human. Our study provides a comprehensive transcriptome-wide view of EJC-RNA interactions in a whole organism and unravels RNA modalities that contribute to the unforeseen biological coherence of the bound transcripts.

RESULTS

Stabilization of the Exon Junction Complex on mRNAs by DSP

The EJC is maintained in its RNA-bound state through direct interaction of the Mago-Y14 heterodimer with the otherwise dynamically binding RNA helicase elf4AIII (Andersen et al., 2006; Bono et al., 2006; Nielsen et al., 2009; Shibuya et al., 2006; Stroupe et al., 2006; Tange et al., 2005). EJC binding to RNA is labile, as under stringent washing conditions (~1 M salt concentrations), interaction of elf4AIII and Mago-Y14 is abolished and the RNA is released from the complex (Singh et al., 2012). We therefore hypothesized that introducing covalent bonds between the Mago-Y14 heterodimer and elf4AIII might stabilize the EJC on its RNA targets and render the protein-RNA complex resistant to the high salt concentrations commonly used in iCLIP (individual-nucleotide-resolution crosslinking and immunoprecipitation) studies. Furthermore, a stabilized EJC complex would enable us to “pull” on EJC subunits other than the RNA-binding elf4AIII, ensuring isolation of the complex under stringent conditions. To test this we made use of the bivalent crosslinking agent dithiobis(succinimidylpropionate) (DSP), which reversibly crosslinks primary amino groups of polypeptides in close proximity (Lomant and Fairbanks, 1976; Schweizer et al., 1982). We isolated poly(A)-containing mRNPs on an oligo d(T)~resin (Castello et al., 2012; 2013) from cytoplasm of adult Drosophila either untreated or treated with UV, DSP, or UV plus DSP (Figure 1). SDS-PAGE silver staining and western blot analyses of mRNA-RNP precipitates (Figures 1A and 1B) revealed that irradiation of cytoplasmic lysates by UV ex vivo only marginally increased co-precipitation of proteins with poly(A)-containing RNAs (Figure 1A, lanes 7 and 8). Upon UV irradiation, only faint signals of known RBPs, such as elf4AIII and cytoplasmic poly(A) binding protein (PABP), were detected in the poly(A) RNA precipitates. Non-RNA-binding EJC subunits such as Y14 were not detected (Figure 1B, lanes 7 and 8, and Figure 1C), in agreement with previous observations (Castello et al., 2012). In contrast, treatment of cytoplasmic lysates with DSP led to strong protein co-precipitation with mRNAs (Figure 1A, compare lanes 7–10). Western blot analysis of precipitates from DSP- and UV-DSP-treated cytoplasmic lysates revealed strong signals not only for direct mRNA binding proteins such as elf4AIII and PABP but also mRNPs components not directly bound to RNA, such as Y14 (compare Figures 1A and 1B, lanes 7–10, and Figure 1C). In none of the precipitates were cytoplasmic proteins such as kinesin heavy chain (Khc) or the small ribosomal subunit protein RpS6 observed (Figure 1B, lanes 7–10, and Figure 1C), confirming the stringency of the assay. Furthermore, precipitates from the beads-only control were free of all proteins tested (Figure 1A, lane 6), indicating that artifacts due to DSP or UV treatment are unlikely. These observations suggest that for EJC stabilization on RNA, DSP-mediated covalent bond formation between individual EJC subunits is superior to UV crosslinking and support the use of DSP when studying other mRNP assemblies (Figure 1D).

ipaRt: An Approach for High-Quality Isolation of EJC Complexes Associated with RNA Templates

Of the tagged EJC subunits we tested, GFP-Mago showed the highest degree of incorporation into endogenous EJCs (Figure S1B). The elf4AIII subunit was additionally found to co-sediment with polysome fractions in sucrose density gradients, independently of Mago-Y14 (Figure S1C), suggesting that the DEAD-box helicase might have yet unknown EJC-independent functions in the fly. Therefore, we carried out EJC-specific RNA immunoprecipitation (RIP) from DSP-treated cytoplasmic extracts prepared from GFP-Mago- and GFP tag-expressing flies. By titrating salt and detergent concentrations, we identified stringent washing conditions (see STAR Methods) that yielded high-quality RNA profiles from GFP-Mago RIPs and only scant RNA profiles from GFP control RIPs, compared with standard IP washing conditions (Figure 2A, compare lanes 2–5). To test if the presence of RNA in GFP-Mago precipitates was a consequence of its incorporation into the EJC rather than by virtue of transient interactions of Mago with other RBPs, we subjected immunoprecipitates from DSP-treated or untreated lysates to RNase digestion. Western analysis revealed the presence of all tested EJC subunits in the GFP-Mago precipitates (Figure 2B, lanes 7 and 8) but no protein other than GFP itself in the GFP RIP controls (Figure 2B, lanes 3 and 4). In contrast, we detected no signal for the proteins probed in the beads-only control precipitates (Figure 2B, lanes 2 and 6) and observed signals for the RNA non-binding Khc only in lysate inputs (Figure 2B, compare lanes 1 and 5, 2–4, and 6–8), indicating high stringency of the assay.

The stabilizing effect of DSP on the EJC is evident from the enhanced elf4AIII signals in GFP-Mago precipitates when cytoplasm was treated with DSP prior to immunoprecipitation (Figure 2B, lanes 5, 7, and 8). Conversely, the PABP signal in the GFP-Mago precipitates disappeared upon incubation with RNase of both DSP-treated and the untreated samples (Figure 2B, lanes 1, 5 2–4, and 6–8). This shows that even when exposed to DSP, proteins whose associations with the EJC are bridged by RNA can be removed by RNA fragmentation. To confirm the “cleansing effect” of RNase, we performed IPs from DSP-treated cytoplasm with or without an RNA fragmentation step and analyzed the precipitates using tandem mass spectrometry (MS). Expression set analysis of MS signals obtained in GFP-Mago and GFP control precipitates identified 45 versus 35 significantly enriched proteins in the untreated and RNase-treated samples, respectively (Figure 2C; Figure S2; Table S4). Although all EJC subunits were enriched independently of RNA integrity (Figure 2C), only upon RNA fragmentation was Btz enriched to a similar degree as Mago, Y14, and elf4AIII. Except for the poly(A) binding protein Nab2 (Bienkowski et al.,
2017), no EJC-unrelated RBPs showed significant enrichment upon RNA fragmentation (Figure 2C), showing that RNA fragmentation by RNaseI increases both sensitivity and specificity of EJC IPs.

We conclude that DSP is a suitable tool for stabilization and isolation of EJC RNA complexes from animal tissues and that our protocol provides a reliable approach for isolation of proteins (or protein complexes) associated with their RNA targets. We termed our experimental strategy “ipaRt.”

EJC Binding in Drosophila Cytoplasm Maps to Canonical Deposition Sites

To determine which sites in the Drosophila transcriptome are protected by the EJC as opposed to other mRNA binding proteins (mRBPs), we performed EJC ipaRt- and oligo(dT)-mediated mRNP capture in parallel, both followed by an RNase digestion step (see STAR Methods), and constructed cDNA libraries of the protein-protected RNA fragments as described for iCLIP (Konig et al., 2011). Analysis of the sequencing results revealed that more than 92% of all reads aligned uniquely to the Drosophila genome (Figure S2B). 85% of EJC ipaRt reads mapped to exons, as opposed to 34% in the mRBP footprinting mapped reads, indicating specificity of the ipaRt library (Figure 3A).

To define the median binding coordinates of the protected sites, we determined the sequence coverage ±50 nt of exon-exon junctions in EJC ipaRt and mRBP footprinting (Figure 3B) and averaged the coverage profile over all junctions. The mean coverage profile in mRBP footprinting appeared evenly distributed, indicating an absence of protection bias (Figure 3B). In contrast, the protected sites in EJC ipaRt were located almost exclusively in upstream exons, with an EJC coverage median –21.7 nt 5’ to the exon-exon junction (Figure 3B), consistent...
Figure 2. DSP Crosslinking Stabilizes EJC for Stringent Immunoprecipitation and RNA Fragmentation

(A) Comparison of IP washing conditions for RNA isolation. Anti-GFP RNA IP from DSP-treated cytoplasm of GFP-Mago- and GFP tag-expressing flies. 0.02% of RNA isolated from input lysate (lane 1) and 20% from GFP tag (lanes 2 and 3) and GFP-Mago (lanes 4 and 5) RIP precipitates were resolved by capillary gel electrophoresis on an RNA 6000 Pico Chip Bioanalyzer. Washing conditions are indicated at bottom. SW, standard-stringency washing conditions; HSW, high-stringency washing conditions (see STAR Methods).

(B) DSP stabilized EJC core is resistant to RNaseI treatment. Effects of RNaseI treatment on GFP-Mago co-immunoprecipitations in DSP crosslinked and untreated cytoplasm. Western blots of anti-GFP IPs from DSP-treated and from untreated cytoplasm of GFP-Mago (lanes 5–8) and GFP tag (lanes 1–4) expressing flies. Precipitates were subjected to isobar labeling, and peptide content was defined by tandem mass spectrometry. Bar plots of protein enrichment are defined by Limma (Ritchie et al., 2015). GFP-Mago-specific protein enrichments in intact RNA and fragmented RNA conditions are highlighted in left and right plots, respectively. The y axis shows individual proteins detected. The x axis shows scale of enrichment (log 2 fold change). Dashed line indicates average enrichment of all significant proteins in each condition. Protein enrichment > 2 times or <2 times average enrichment is indicated by solid or transparent bars, respectively. Enrichment bar color legend is highlighted at the bottom. ns, non-significant (adjusted p value [p.adj.] > 0.05).

(C) RNA fragmentation depletes EJC-unrelated proteins from GFP-Mago co-precipitates. Anti-GFP IPs (HSW condition) from DSP-treated cytoplasm of GFP-Mago- and GFP tag-expressing flies processed under HSW conditions. Primary antibodies used are indicated on the right. Inputs (0.01%) are shown in lanes 1 and 5. Bead-only control precipitates were located remotely (>50 nt) of canonical EJC binding regions (Figures 3D, 3G, and S4A).

Protection Sites Remote of Exon-Exon Junctions in EJC

Studies of mammalian EJCs have reported a high frequency of EJC-mediated protection outside of canonical binding sites (non-canonical EJC deposition sites) (Saulière et al., 2012; Singh et al., 2012). To test whether this non-canonical distribution is representative of EJC protection across the Drosophila transcriptome, we determined coverage maxima for every exon-exon junction protected by EJC. The majority (~95.5%) of protection peaks in EJC ipaRt libraries mapped to sites of canonical EJC binding, proximal to exon-exon junctions (Figures 3C and 3D). The remaining ipaRt protection coverage peaks were located remotely (>50 nt) of canonical EJC binding regions (Figures 3D, 3G, and S4A).

Our analysis shows that proximal peaks map mainly to internal exons (79%), to first exons (20.6%), and only minimally to terminal exons (0.4%), as expected given the splicing-dependent deposition of EJCs upstream of splice junctions. Remote
Figure 3. EJC Binding to mRNA in *Drosophila* Cytoplasm Occurs within Exons at Canonical Deposition Sites

(A) EJC ipaRt library reads map to exonic sites in the *Drosophila* genome. Summary of genomic features detected in mRBP footprinting and EJC ipaRt sequencing results. The y axis indicates the proportion (percentage) of uniquely aligning read counts. Color code of genomic features highlighted in the legend on right side of the plot. Note that EJC-protected sites map in majority to exons and UTR exons.

(B) EJC protection median is on upstream exons approximately −22 nt from the 3' end. Read coverage profiles from EJC ipaRt and mRBP footprinting cDNA libraries. Coordinates of metagene covering +50 nt of exon-exon junctions are indicated on x axis. Note that position 0 defines the last nucleotide of upstream exons.

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EJC binding. Consistent with this hypothesis, analysis of EJC and RBP protection sites on Drosophila transcripts annotated in the FlyFISH RNA localization database (Lécuyer et al., 2007; Wilk et al., 2016) revealed that localized maternal mRNAs are more likely to be EJC bound than non-localizing transcripts (Figure 4C).

**Gene Architecture Determines EJC Assembly on mRNAs**

It was reported that in mammalian cells, EJCs are enriched on mRNAs from alternatively spliced genes (Hauer et al., 2016). In the fly, the EJC has been reported to promote correct splicing of long intron-containing genes (Ashton-Beaucage et al., 2010; Roignant and Treisman, 2010). This relationship between EJCs and gene architecture led us to ask which features could explain the gene-to-gene variation in EJC deposition. We used a multiple regression model (see STAR Methods) to assess how five features (number of introns, maximum intron length, transcript abundance, transcript length, and the degree of alternative splicing) influence gene deposition of EJC. We checked that the effects estimated from the model hold given underlying correlations of the features. For example, after accounting for intron number, which has a strong effect on EJC binding (Figure S5A), we determined that intron length also has a strong positive effect (Figure S5B), while alternative splicing has only a minimal effect on EJC binding (Figure S5C).

It is noteworthy that exon-exon junctions of transcripts from genes comprising at least one large intron (≥10,000 bp) were significantly more enriched than those of genes lacking large introns (Figure S5F). Within transcripts of long intron-containing genes, EJC assembly was biased neither toward junctions formed upon large-intron splicing nor toward neighboring junctions (Figure S5G). Instead, exon-exon junctions within these transcripts showed a general elevation in EJC binding.
Figure 4. Preferential Recruitment of EJC to mRNAs of Genes with Specialized Cellular Functions Is Determined by Gene Architecture

(A) MA plot of DESeq results from EJC ipaRt and mRBP footprinting. Genes that are either enriched or depleted for EJC (RBP enriched) are indicated in red or gray, respectively. Genes not significantly different (p.adj. > 0.05) between the EJC ipaRt and mRBP libraries are transparent. Relative enrichment (log 2-fold change) is indicated on the y axis. Base mean of signal is highlighted on the x axis. Dashed line defines log 2 fold change = 0.

(B) GO term analysis of EJC-enriched and EJC-depleted transcripts. GO terms for biological processes of EJC enriched transcripts (left plot) and of EJC depleted transcripts (right plot) are presented to the left and the right of the plots, respectively. The y axis list GO categories of the biological processes most highly represented. Gene counts in individual categories versus overall count of analyzed genes (gene ratios) are shown on the x axis. Legend indicating

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probability, pointing to a “global” large intron-mediated effect on EJC assembly (Figure S5G).

Finally, we assessed the relative importance of each of the five features from the multivariate regression analysis (Figure 4D). Two features dominated our model of EJC deposition, the number of introns per gene and the maximum intron length of the gene (Figure 4D), are positively associated with EJC deposition. This indicates that a gene’s architecture is a main determinant of EJC binding.

**Splice Site Strength and Hexamer Composition Influence EJC Deposition**

We estimated EJC enrichment at the junction level using reads within ±50 nt of the splice site and observed a strong dependency on the gene EJC estimate (Pearson correlation = 0.66, \( p < 2e-16 \)). This suggests that the junction’s EJC profile is primarily determined by its parent gene architecture. We next tested whether any exon-exon junction deviates significantly from its parent gene EJC binding. About 31% of detected junctions have an enrichment that deviates significantly from the gene level (Figure 5A). Furthermore, we observed that EJC reads cover only a subset of junctions within a gene and show higher read coverage coefficient of variation than reads protected by other RBPs (Figures S5D and S6E).

We asked if a known sequence context related to splicing might be responsible for the variability in EJC binding to exon-exon junctions within a gene. We found that strong 5′ and 3′ splice site signals and the presence of 5′ intronic splicing enhancers (5′IEs) correlate with increased EJC deposition, while the presence of an 5′ exonic splicing silencers (5′ESSs) correlates with reduced EJC deposition (Table S1; Figure 7A), consistent with the fact that EJC assembly is dependent on splicing. Surprisingly, 5′ and 3′ exonic splicing enhancers (ESEs) and intronic ISEs at 3′ splice sites have no effect. Next, we tested the effects of unannotated hexamers, while accounting for ESS, ISE, and splice strength. We detected 63 hexamers associated with a change in EJC deposition. By clustering the hexamers on the basis of sequence similarity, two major groups with either a negative effect or a positive effect on EJC assembly emerged (Figure 5B). Strikingly, 5 of the 16 hexamers associated with increased EJC deposition contain the trinucleotide CUG, and 28 of the 47 hexamers associated with decreased EJC deposition contain the trinucleotide UUU (Figure 5B). The strongest effect of these CUG or UUU trinucleotides is observed when they are present around the region downstream of EJC binding (approximately −16 to −18 nt). This indicates that the sequence composition of this region is a strong determinant of EJC binding (Figure 5C).

**RNA Structure Modulates the Degree and Position of EJC Binding in Drosophila**

Deposition of an EJC at the first exon-exon junction and presence of a structured element next to the deposition site are required for localization of oskar mRNA at the posterior pole of the *Drosophila* oocyte (Ghosh et al., 2012; Simon et al., 2015). Interestingly, we observed 2.38-fold stronger enrichment of the first oskar exon-exon junction than anticipated from oskar mRNA enrichment in our data, indicating that structures near EJC binding sites might affect EJC assembly.

To test if RNA structures might affect EJC binding, we estimated for every junction the probability of base-pairing for each nucleotide −37 to +28 bp of the splice site. We observed three distinct average base-pairing probability (bpp) profiles for exon-exon junctions with unaffected, positively correlated, or negatively correlated EJC binding (Figure 6A). Two regions showed significantly different bpps for junctions with a positive versus a negative effect on EJC binding (Figure 6B). The first region, located in the canonical EJC binding site, showed a decreased bpp for junctions with a positive EJC binding effect (Figures 6A and 6B) and increased bpp for junctions with negative EJC binding effect (Figures 6A and 6B). Surprisingly the second region, located directly downstream of the canonical deposition site (Figures 6A and 6B), showed an elevated bpp near junctions with positive EJC binding but decreased bpp at junctions with negative EJC binding (Figure 6A). This result indicates that although EJC binding in *Drosophila* occurs on single-stranded RNA (ssRNA), in agreement with previous reports (Andersen et al., 2006; Bono et al., 2006), EJC binding to RNA may be enhanced by RNA secondary structures proximal to the EJC binding site.

Given the redundant information between bpp of each nucleotide pair, we performed dimension reduction on bpp profiles within the −24 to −11 region (STAR Methods; Figure 6B) using a Gaussian mixture model, to facilitate subsequent analysis of EJC binding. We obtained four folding categories (Figure 6C) and observed an association between significant positive EJC binding (log₂ fold change > log₂[1.5]) and junctions harboring folding categories 2 and 3, which contain a bpp elevation downstream of, or surrounding, EJC binding sites (Figures 6C and 6D). Junctions with an unstructured profile (folding category 1) show no such bias, and junctions with bpp elevation in the EJC binding site (folding category 4) are associated with a negative EJC
binding effect. This suggests that when located near EJC deposition sites, RNA structures may positively affect EJC binding in *Drosophila* (Figures 6C and 6D, lanes 2 and 3) and could explain the enhanced binding of EJC to the first exon-exon junction in *oskar* mRNA.

Previous in vitro experiments have shown that RNA secondary structures affect EJC assembly site coordinates (Mishler et al., 2008). We asked whether predicted stable RNA stem structures within or flanking a canonical EJC binding site at exon-exon junctions have any impact on the precise coordinates of EJC assembly in the fly. For this we estimated potential double-stranded RNA (dsRNA) content in region A, spanning from −30 to −21 and for region B spanning from −23 to −14 within exon-exon junctions (see sketch in Figure 6E). We focused on junctions with a positive EJC binding effect to allow robust estimates on the basis of junctions with high coverage. The analysis revealed a strong downstream shift of EJC assembly coordinates when dsRNA content in region A was high and low in region B, and only a minor shift when the dsRNA content was low in region A and high in region B (Figure 6F). Taken together, these
Figure 6. mRNA Secondary Structures Modulate EJC Binding
(A) Pairing probability profiles of EJC bound exon-exon junctions. Predicted base-pairing probability (bpp) profiles, by RNAplfold from Vienna RNA package, of junctions with enhanced, inhibited, and unaffected EJC binding. Black dashed, red, and gray solid lines highlight average bpp profiles of junctions that are unaffected, enhanced, and inhibited for EJC binding, respectively. Note that the region used for RNA-fold analysis covers the last 37 nt of the upstream exon and the first 28 nt of the downstream exon. The y axis shows predicted bpp. The x axis highlights nucleotide positions relative to exon-exon junction. Position 0 represents last RNA nucleotide of upstream exons. Dashed vertical line highlights the coordinate (~21.7) of the average EJC protection median.

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observations confirm that the structural context of exon-exon junctions not only affects EJC binding efficiency but also directs the site of EJC assembly.

**Comparison of Human and Drosophila Datasets Reveals Common Factors that Influence EJC Enrichment**

Previous studies of the EJC in *Drosophila* and human reported differences between these two species in terms of EJC protein components and cellular function. We asked whether any of the factors we identified in *Drosophila* would also influence EJC deposition in human cell lines. Using the tools and models described in previous sections, we analyzed CLIP enrichment of the EJC component BTZ in HeLa cells (Hauer et al., 2016). Results from the multiple regression analysis showed that the number of introns is a major determinant of the gene-to-gene variation in EJC deposition (Figure S7A). This agrees with our finding in *Drosophila* and suggests that this mechanism is conserved between *Drosophila* and humans. In contrast to our observations in the fly, we found that in mammals the extent of alternative splicing in genes has a small but positive effect on EJC deposition (Figure S7A), in agreement with previous findings that alternatively spliced genes are over-represented in EJC-enriched genes (Hauer et al., 2016; Saulière et al., 2012). However, in contrast to *Drosophila*, in humans intron length did not facilitate but rather antagonized EJC mRNA binding (Figure S7A).

Next, we investigated the factors that determine variability in EJC deposition within genes (Table S2; Figure S7D). Similar to our *Drosophila* findings, high 5' and 3' splice strength and presence of an ISE at the 5' junction enhance EJC deposition within a junction. In this analysis, presence of ESEs in the upstream and of ESS in the downstream 50 nt strongly affects EJC deposition, something we did not observe in *Drosophila* (Figure 7A). A possible explanation for this is that ESE, ESS, and ISE sequences are annotated based on experiments in human cell lines and may not exert a similar effect in *Drosophila*. Among the 238 ESEs, we observed 28 hexamers containing AGAA, which is similar to a motif (GAAGA) found in a previous EJC CLIP study (Hauer et al., 2016; Saulière et al., 2012). We separated ESEs according to the presence of AGAA and indeed found that the ESEs containing AGAA are associated with stronger EJC deposition. We asked whether bpp profiles differ between junctions with positive and negative EJC binding. We found that an overall negative bpp (−24 to −18) around the EJC deposition site favors EJC deposition (Figures S7B and S7C). Unlike *Drosophila*, we did not find other regions whose bpps are associated with increased EJC deposition in mammalian cells. Taken together, these results indicate that across *Drosophila* and human, not only do conserved regulatory mechanisms such as intron counts, splice strength, or structural hindrance within EJC deposition sites influence EJC deposition, but also divergent regulatory factors such as ESEs in mammals or RNA folding in proximity of EJC deposition sites in *Drosophila* can affect EJC deposition.

**Features that Inform on EJC Binding May Predict mRNA Localization**

The bias of EJC binding to mRNAs from genes with functions in development and cell polarity suggested that EJC binding might be indicative of transcripts under spatial or temporal control. Consistent with this hypothesis, analysis of EJC and RBP protection sites on *Drosophila* transcripts annotated in the FlyFISH RNA localization database (Lécuyer et al., 2007; Wilk et al., 2016) revealed that localized maternal mRNAs are more bound by EJC than non-localizing transcripts (Figure 4C). We postulated that modalities underlying EJC enrichment at the gene and junction level can inform us about the localization of a transcript and applied decision tree learning on RNA localization using the R package rpart (Therneau and Atkinson, 2018). Given the imbalance between localized to non-localized dataset, we used Cohen’s kappa coefficient to assess the predictive value of the model using different data groups. The use of gene features and features of the most enriched EJC junction is sufficient to achieve predictive accuracy comparable with a model.
Figure 7. Summary of All Factors Regulating EJC Assembly at Exon-Exon Junctions in Drosophila

(A) Factors in Drosophila that determine EJC binding variability within a transcript. Plot comparing the effects of the different variables on junction level EJC enrichment in Drosophila. We fitted a full linear model: Δlog₂ fold change ~ splice site (SS) strength + ESE + ESS + ISE + hexamer categories + folding categories. Δlog₂ fold change is the difference between junction EJC enrichment and gene EJC enrichment. 5SS and 3SS would be the splice strength present at 5' and 3' splice junctions. Hexamer and folding categories were defined in Figures 5B and 6C, respectively. ESE, ESS, and ISE stand for the number of indicated splicing regulatory elements present in the upstream exon or downstream intron of splice site, respectively.

(B) Model of EJC assembly and variable binding along the gene's transcript. Co-transcriptional recruitment is affected by speed of mRNA production. mRNAs of simple genes with low complexity and small number of introns (n) are transcribed and processed faster than mRNAs from genes with diverse intron sizes and large number of introns. Longer processing increases retention time (Δt) of assembled spliceosomes at the site of transcription and thereby increases the likelihood of EJC assembly \( L_{EJC} = n \times M \), where \( M \) is the number of splicing events and \( n \) is the retention time of spliceosomes at active RNA pol II sites.

(C) Cohen’s kappa coefficient for different models. Box plots showing the distribution of Cohen’s kappa coefficient obtained after fitting tree model for classification by recursive partitioning (over 100 bootstraps of four different data groups) using R package “rpart” (http://CRAN.R-project.org/package=rpart) (Therneau and Atkinson, 2018). Group 1 includes gene features of the mRNA that influence EJC deposition from Figure 4E. Group 2 includes junction features (from Figure 7A) of the junction with the highest EJC deposition. Group 3 includes all variables in groups 1 and 2. Group 4 includes all the variables from group 3 and EJC estimates (Δlog₂ fold change for the gene and highest EJC deposited junction and the delta value). Median values are 0.246 (data group 1), 0.267 (group 2), 0.306 (group 3), and 0.315 (group 4).

(D) Relative variable importance for all features. Box plots showing the distribution of variable importance for all variables obtained after fitting tree model for classification, rpart over 1,000 bootstraps of data group 3. The variable importance is an estimate of the usefulness of the variable in splitting the different classes in the regression tree. Highlighted in red are variables that are likely more useful in the classification compared with the other variables.
incorporating all possible features (Figure 7C). Using the gene and highest EJC covered junction information as a working model, we asked which variables are important in this model. To prevent bias in estimate, the data were bootstrapped 1,000 times. We observed that transcript length, maximum intron size in the gene, and 5′ splice strength and folding of the most enriched junction are more useful predictors (Figure 7D) compared with other variables. This suggests that features of gene architecture that orchestrate EJC deposition can distinguish localizing and non-localizing mRNA and might be important for mRNA localization.

DISCUSSION

ipaRt: A Method for High-Confidence Identification of Protein Binding Sites on RNAs In Vivo

We have profiled the landscape of EJC binding across the transcriptome of a whole animal, Drosophila melanogaster, and determined the parameters that influence the distribution of the complex on RNAs in the organism. Previous knowledge of EJC-RNA interactions was based on UV-crosslinking experiments in specific cell types grown as homogeneous cultures for the individual studies (Hauer et al., 2016; Ince-Dunn et al., 2012; König et al., 2010, 2011; Licatalosi et al., 2008; Modic et al., 2013; Saulière et al., 2012; Tollervey et al., 2011; Ule, 2009; Ule et al., 2003; Wang et al., 2010). Although UV crosslinking remains a method of choice for identification of protein binding sites on nucleic acids, because of the inefficient penetration of UV light into tissues and organisms, the method is most useful when applied to cells in culture. In contrast, our analysis of EJC distribution in the tissues of whole Drosophila flies was made possible by ipaRt, which uses the crosslinking agent DSP to freeze protein-protein interactions within otherwise dynamic RNP complexes, such as the EJC.

We have demonstrated that DSP-mediated covalent bond formation between the RNA helicase elf4AII and the Mago-Y14 heterodimer preserves EJCs in their “locked” state on mRNAs (Andersen et al., 2006; Ballut et al., 2005; Bono et al., 2006; Stroupe et al., 2006) and that efficient recovery of the bound RNAs does not require their crosslinking to elf4AII using UV light. Our “ipaRt” approach, like CLIP and iCLIP (König et al., 2010; Ule et al., 2003), enables highly stuttering washing of the samples. In support of the robustness and reliability of our DSP-based assays, we demonstrated high reproducibility not only among technical but also biological replicates of EJC ipaRt, as well as mRBP footprinting sequencing results (Figure S4A).

Furthermore, ipaRt allows the use of non-RNA-binding subunits of the EJC, such as Mago, as immunoprecipitation baits. This is highly relevant in the context of the EJC, as we and others have shown that its RNA-binding subunit, the RNA helicase elf4AII, may have other, EJC-independent functions in the cell (Figure S1C) (Alexandrov et al., 2011; Choudhury et al., 2016). ipaRt afforded us the option of using Mago as our EJC bait, and indeed this is a main reason for the high-quality definition of the EJC binding landscape in the fly cytoplasm that we have achieved. The protection site reads we obtained from EJC ipaRt map almost exclusively to canonical EJC deposition sites (Le Hir et al., 2000) with a median protection ~22 nt of the upstream exon’s 3′ end. In contrast to mammalian EJC CLIP and RIP studies, in which elf4AII served as an immunoprecipitation bait (Saulière et al., 2012; Singh et al., 2012), EJC ipaRt reads mapping to regions distant from canonical deposition sites are of low abundance and sequencing coverage. Although this discrepancy could reflect differences in EJC engagement in humans and Drosophila, it more likely reflects the choice of bait or the cell compartment in which the analysis was executed. Indeed, a recent study in human cells revealed that when the cytoplasmic EJC component Btz was chosen as the bait rather than elf4AII, the proportion of non-canonical EJC deposition sites was negligible (Hauer et al., 2016).

Finally, in ipaRt the DSP crosslinker is applied ex vivo during tissue disruption and does not require inhibition of translation in vivo. We therefore consider ipaRt a method of choice for functional investigations of protein-RNA complexes in fully developed organisms and tissues.

Regulation of EJC Assembly in Drosophila

Through our analysis, we defined factors that contribute to or inhibit EJC assembly on mRNAs and at individual exon-exon junctions in Drosophila. From this we deduce that the landscape of EJC binding to RNAs is sculpted through regulation of EJC assembly at two levels in the fly (Figure 7B).

At the upstream regulatory level, the degree to which EJCs are assembled on an mRNA is dictated by the complexity of the gene’s architecture: mRNAs produced from genes of simple architecture are marked by fewer EJCs, while mRNAs from genes of complex architecture, comprising multiple splice sites and long introns, are EJC bound to a higher degree (Figures 4D and S6B). Given that EJCs assemble on mRNAs concomitantly with splicing (Alexandrov et al., 2012; Barbosa et al., 2012; Le Hir et al., 2000; Steckelberg et al., 2015), it is not surprising that mRNAs of genes containing a greater number of introns are more likely to be EJC bound. However, our finding that the enhancing effect on EJC binding provoked by large introns is not restricted to flanking junctions but occurs at junctions mRNA-wide is unexpected (Figure S5F). Loss-of-function experiments indicate that the EJC participates in exon definition during splicing of long intron-containing genes in Drosophila (Ashton-Beaucage et al., 2010; Rognant and Treisman, 2010), particularly in definition of exons proximal to the long introns (Hayashi et al., 2014; Malone et al., 2014). Our data exclude any significant bias toward EJC assembly in proximity to long-intron splice junctions. Instead they reveal a general enhancement of EJC binding at exon-exon junctions throughout transcripts of long-intron genes (Figure S5G). Therefore, we conclude that stable binding of EJCs within mRNAs of long-intron genes is not the result of EJC engagement in exon definition. Instead, we propose that the high degree of EJC binding to long-intron transcripts derives from the increased number and resting time of co-translationally assembled spliceosomes on the nascent transcripts, which would increase the probability of CWC22-dependent elf4AII recruitment to pre-mRNAs during splicing (Figure 7B).

At the downstream regulatory level, after EJC assembly rates at transcripts are defined, deposition of EJCs along mRNA exon-exon junctions is modulated by the structural and sequence context of the splice sites (Figures 7A and 7B). dsRNA stem
structures in exon-exon junctions of Drosophila mRNAs either antagonize EJC assembly when present within canonical EJC deposition sites or enhance EJC assembly when located in the vicinity of the EJC deposition site (Figures 4D and 7A). Absence of dsRNA within the EJC binding moiety is in agreement with reported preference of EJCs for ssRNA (Andersen et al., 2006; Bono et al., 2006; Mishler et al., 2008). It remains to be elucidated how and why EJC binding is positively affected when RNA stem structures are found in its direct proximity on the bound template.

Although it is likely that the structural context of exon-exon junctions in Drosophila directly influences the degree of EJC assembly, sequence composition-derived effects on EJC binding to mRNA are a consequence of the assigned roles of these sequences during pre-mRNA splicing. We have demonstrated that exon-exon junctions with strong 5’ and strong 3’ splice sites (SSs) are biased toward junctions with enhanced EJC binding (Table S1; Figure 7A). For the regulation of weak 5’ and 3’ SSs, which commonly occur at alternatively spliced junctions, cis-acting splicing regulatory elements (SREs) were shown to be of importance (Brooks et al., 2011; Koren et al., 2007; Shepard et al., 2011). In light of the negative impact of alternative splicing at the level of EJC mRNA binding (Figure 4D), it is not surprising that conventional ESEs and ESSs hardly affect EJC binding at the level of individual exon-exon junctions. Whether the position-dependent bias mediated by the UUU-triplet- and CUG-triplet-containing hexamers toward inhibited or enhanced EJC binding that we have discovered in our Drosophila dataset (Figures 5B and 5G) is due to a direct or indirect influence of these hexamers on splicing remains to be addressed. UUU-triplet-containing hexamers, which are strongly biased against EJC binding, could potentially function as yet undefined 5’ESS in Drosophila. Interestingly, CUG-triplet-containing hexamers, which are strongly biased toward enhanced EJC binding, share sequence similarity with a previously predicted CUG containing 5’ESE of short intron splice sites (Brooks et al., 2011). It appears likely that the CUG-triplet and UUU-triplet hexamers exert their effect on EJC binding as a yet undefined class of SREs.

RNA Modalities Influencing EJC Binding in Mammals and Fly

In agreement with reports in mammals (Hauer et al., 2016; Saulière et al., 2010, 2012; Singh et al., 2012), the extent of EJC occupancy varies between mRNAs and exon-exon junctions also in Drosophila. The splice site score next to a junction correlates with increased EJC deposition in the fly, and this relationship between splicing efficiency and EJC deposition has also been proposed in mammalian studies (Custódio et al., 2004; Gudikote et al., 2005). Analysis of published mammalian Btz iCLIP data (Hauer et al., 2016) revealed several modalities that correlate with the increased binding landscape of the EJC on mRNAs in both mammals and Drosophila, including the large number of introns, high transcript abundance, and sequence context of individual exon-exon junctions (Figure S7). Interestingly, the presence of long introns has a slightly negative effect and the amount of alternative splicing a slightly positive effect on EJC occupancy in mammals (Figures 4D and S7); the latter agrees with previous observations (Hauer et al., 2016; Saulière et al., 2012; Singh et al., 2012). Studies in cultured mammalian cells have reported that EJC-enriched junctions contain a relatively high proportion of “non-canonical” protection sites, which were enriched for RBP consensus sequences of the SR protein family (Saulière et al., 2012; Singh et al., 2012). Our analysis of mammalian Btz iCLIP data (Hauer et al., 2016) confirms that presence of ESEs in upstream exons and 5’ISEs in introns correlates with enhanced EJC binding (Table S2). Moreover, we have identified a group of junctions in mammals containing AGAA hexamers that are biased for enhanced EJC binding (Figure S6A), but their effects are not especially strong near the canonical EJC deposition site (Figure S6B). These hexamers match the AGAA-encompassing consensus sequence of the mammalian SR protein SRSF10, known to function as splicing enhancers (Cléry et al., 2011; Tsuda et al., 2011), and have been found previously in EJC bound exon-exon junctions (Hauer et al., 2016; Saulière et al., 2012; Singh et al., 2012). Not only do our in silico results agree with these reports and support the proposed cooperative binding of EJC with SR proteins (Hauer et al., 2016; Saulière et al., 2012; Singh et al., 2012), they also partially explain the EJC’s preference in mammals for alternatively spliced mRNAs.

One observation deriving from our analysis of published mammalian Btz iCLIP datasets is surprising. Although we observed junctions in Drosophila to be enhanced or inhibited in EJC binding by specific base-pairing probability (bpp) profiles, thus by specific RNA folding categories (Figures 6A–6D), we could not detect any striking difference between overall bpp profiles of exon-exon junctions with enhanced or inhibited EJC binding in mammals (Figure S7B). Indeed, the only aspect of RNA structure shared by mammals and Drosophila is the negative effect of dsRNA when directly overlapping with the canonical EJC deposition site (Figure S7C) (Mishler et al., 2008). In Drosophila, however, the presence of dsRNA close to canonical deposition sites enhances EJC binding, an effect that is not observed in mammalian cells.

Insights into Evolution and Divergence of EJC Functions

Our findings regarding the differences in the RNA modalities enriched at highly occupied mammalian and Drosophila EJC sites provide insight into the expansion of functions of the EJC during eukaryotic evolution. Spliceosome catalyzed splicing reactions are bidirectional, and efficient formation of exon-exon junctions during RNA maturation is achieved by Prp22-induced release of spliceosomes from mRNAs (Hoskins and Moore, 2012; Smith and Konarska, 2008; Tseng and Cheng, 2008). The EJC is absent in organisms with low rates of RNA splicing, such as Saccharomyces cerevisiae, but present in organisms with high splicing rates, such as Schizosaccharomyces pombe (Goffeau et al., 1996; Wen and Brogna, 2010; Wood et al., 2002). This suggests that with the increased demand for splicing accuracy in higher eukaryotes, the EJC evolved to function as an exon-exon junction “lock” hindering spliceosome reassembly at spliced exon-exon junctions. Because EJC binding in the fly is enhanced at strong splices sites, but is not affected by splicing enhancer elements, and is not biased toward alternatively spliced mRNAs, we propose that the EJC preserved its primary function as such a lock in Drosophila. Two recent studies provide evidence that in mammals bound EJC’s hinder spliceosome assembly...
suppressing recursive splicing (RS) of RS exons. The previously reported importance of EJC for splicing fidelity (Hayashi et al., 2014; Malone et al., 2014), and our observations on the mode of EJC binding to transcripts in the fly revealing its independence from splicing regulatory elements indeed supports that the EJC’s most conserved function is to ensure splicing irreversibility.

The EJC further evolved to become a central component of the NMD pathway (Buchwald et al., 2010; Gehring et al., 2005; Meleiro et al., 2012; Okada-Katsuhata et al., 2012; Palacios et al., 2004; Shibuya et al., 2006; Singh et al., 2007) in mammals, in which more than 95% of all genes are alternatively spliced (Gromadzka et al., 2016). This may explain why EJCs in mammals are enriched on alternatively spliced transcripts. In Drosophila, in which only 30% of all genes appear to be alternatively spliced (Gibilisco et al., 2016), the EJC is not a component of the main NMD pathway (Behm-Ansmant et al., 2007). We propose that although the EJC-NMD pathway evolved before segregation of the proto- and deuterostome clades, it gained importance by complementing the faux 3'UTR-NMD pathway during the evolution of vertebrates (Eberle et al., 2008), for which RNA surveillance and spatiotemporal control of gene expression are essential.

Similarly, recruitment of the EJC and interacting proteins upon splicing to facilitate mRNA localization so far seems exclusive to Drosophila. Two Drosophila-specific features that modulate EJC binding, namely, the presence of a large intron within a gene and secondary structure near the junction, are also predictive of mRNA localization. Although the precise strength of association between these features and mRNA localization remains to be verified with larger and more quantitative datasets, previous studies with the SOLE in oskar RNA have shown that RNA structure and EJC binding are indeed crucial for oskar mRNA localization (Ghosh et al., 2012).

STAR METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

A.O. conceived, designed, and performed wet lab experiments. A.O., G.L., and N.H. contributed reagents, materials, and analysis tools. A.O., G.L., A.O., and N.H. conceived and designed the in silico analyses. A.O., G.L., N.H., A.O., and A.E. analyzed and interpreted the results. A.O., G.L., N.H., and A.E. contributed to the writing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE                                      | SOURCE                                      | IDENTIFIER      |
|----------------------------------------------------------|---------------------------------------------|-----------------|
| **Antibodies**                                           |                                             |                 |
| Rat polyclonal anti-Y14(Drosophila)                      | Laboratory of M. Blanchette                | N/A             |
| Rabbit polyclonal anti-Mago(Drosophila)                  | Laboratory of M. Blanchette                | N/A             |
| Rabbit polyclonal anti-efF4AII(Drosophila)               | Laboratory of I. Palacios                  | N/A             |
| Rabbit polyclonal anti-PABP (poly A binding protein)     | Laboratory of M. Hentze                    | N/A             |
| Rabbit polyclonal anti-RpL32 (ribosome large subunit protein L32) (Drosophila) | Laboratory of M. Hentze                    | N/A             |
| Mouse monoclonal anti-RpS6 [clone: 54D2] (ribosome small subunit protein S6) | Cell Signalling                            | Cat#2317        |
| Rabbit polyclonal anti-GFP                               | Laboratory of T. Pines                     | N/A             |
| Rabbit polyclonal anti-KHC (kinesin heavy chain)         | Cytoskeleton                                | N/A (discontinued) |
| Mouse monoclonal anti-cMyc [clone: 9E10]                 | Santa Cruz Biotechnology                    | Cat#SC-40       |
| Mouse monoclonal anti-Flag M2                            | Sigma Aldrich                              | Cat#F3165       |
| **Chemicals, Peptides, and Recombinant Proteins**        |                                             |                 |
| Dithio(bis-)sucinimidylpropioniate (DSP)                  | Thermo Fisher                              | Cat#22585       |
| Trizol LS                                                | Thermo Fisher                              | Cat#10296010    |
| Heparin                                                  | Sigma-Aldrich                              | Cat#H4784       |
| Western Blocking Reagent                                 | Roche                                      | Cat#11921681001 |
| Lithium dodecyl sulfate                                  | Sigma-Aldrich                              | Cat#L9781       |
| IGEPAL CA-630                                            | Sigma-Aldrich                              | Cat#3021        |
| CompleteMini Protease Inhibitor Cocktail                 | Roche                                      | Cat#11836170001 |
| Ribolock                                                 | Fermentas                                  | Cat#EO0381      |
| RNaseOUT                                                 | Thermo Fisher                              | Cat#10777-019   |
| RNAsel                                                   | New England Biolabs                        | Cat#M0243S      |
| TurboDNase                                               | Thermo Fisher                              | Cat# AM239      |
| T4 RNA Ligase 2 (truncated)                              | New England Biolabs                        | Cat#M0242S      |
| CircLigase II                                            | Epicentre                                  | Cat#CL9025K     |
| PEG400                                                   | Sigma-Aldrich                              | Cat#8074850050  |
| Linear Acrylamide                                        | Thermo Fisher                              | Cat#AM9520      |
| Protein A Agarose                                        | Roche                                      | Cat#11719408001 |
| Protein G Agarose                                        | Roche                                      | Cat#11719416001 |
| GFP trap Agarose                                         | Chromotek                                  | Cat#gta-100     |
| Anti-Flag Agarose                                        | Sigma-Aldrich                              | Cat#A1205       |
| Anti-cMyc Agarose                                        | Sigma-Aldrich                              | N/A             |
| oligo-dT<sub>25</sub> coated magnetic Dynabeads (for mRNA Seq) | Thermo Fisher                              | Cat#61002       |
| oligo-dT<sub>25</sub> Magnetic Beads (for mRBP pulldown assay) | New England Biolabs                        | Cat#S1419S      |
| Amicon Ultra 10K                                         | Merck Millipore                            | Cat#UFC201024   |
| MiniElute Gel Extraction kit                             | Qiagen                                     | Cat#28604       |
| QIAquick PCR purification kit                            | Qiagen                                     | Cat#28104       |
| **Critical Commercial Assays**                           |                                             |                 |
| Illumina TruSeq RNA Sample Preparation v2 Kit            | Illumina                                   | Cat#RS-122-2001 |
| TMT10plex Isobaric Labelling                             | Thermo Fisher                              | Cat#A34808      |
| SuperScript III Reverse Transcriptase Kit                | Sigma-Aldrich                              | Cat#18080044    |
| Phusion Flash High-Fidelity PCR Master Mix                | Thermo Fisher                              | Cat#F-548L      |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Gateway LR Clonase Enzyme Mix | Thermo Fisher | Cat#11791-019 |
| pENTR/D-TOPO Cloning Kit | Thermo Fisher | Cat#K240020 |

**FASTQ files of ipaRt, mRBP footprinting and mRNA-Seq** library sequencing are deposited at European Nucleotide Archive (ENA) and have accession number PRJEB26421.

**Deposited Data**

- **Drosophila melanogaster**: reference proteome (UP000000803) ProteomeID: UP000000803
  - UniProt
  - ftp://ftp.uniprot.org/protomomes/UP000000803

- **Drosophila melanogaster**: reference genome (fasta file)
  - Ensembl
  - ftp://ftp.ensembl.org/pub/release-81//fasta/drosophila_melanogaster/dna/Drosophila_melanogaster.BDGP6.dna_sm.toplevel.fa.gz

- **Drosophila melanogaster**: reference annotation (gtf file)
  - Ensembl
  - ftp://ftp.ensembl.org/pub/release-81//gtf/drosophila_melanogaster/Drosophila_melanogaster.BDGP6.81.gtf.gz

**Experimental Models: Organisms/Strains**

- **D. melanogaster**: wildtype [w1118]
  - Bloomington Drosophila Stock Center BDSC:3605 Flybase ID: FBst003605

- **D. melanogaster**: ubiquitous GFP expression (stable line)
  - Bloomington Drosophila Stock Center BDSC:4888 Flybase ID: FBst004888

- **D. melanogaster**: GFP-Mago expression (stable strain)
  - Newmark et al., 1997 N/A

- **D. melanogaster**: [3A2] UAS mediated FLAG-HA eIF4AII and MYC-Y14 expression (unstable line)
  - This paper N/A

- **D. melanogaster**: UAS mediated Flag-Myc-eGFP expression (unstable line)
  - This paper N/A

**Oligonucleotides**

- eGFP CDS F: caccATGGTGAGCAAGGGC
  - This paper N/A

- eGFP CDS R: CTTGTACAGCTCGTCCATGC
  - This paper N/A

- Y14 CDS F: caccATGGCCGATGTGTTGGACATTG
  - This paper N/A

- Y14 CDS R: TCTGCGACGCTTTTCGGACTT
  - This paper N/A

- 5' pre-Adenylated - L3 App adapter (HPLC purified)
  - Konig et al., 2011 N/A

- CUT oligo (HPLC purified): GTTCAGGATCCACGACGCTCTTCAaaa
  - Konig et al., 2011 N/A

- P3 primer (HPLC purified): CAAGCAGAAGACGGCATACGAGATCGTCTCGGCTTCTGCTGAACCGCTCTTCGATCT
  - Konig et al., 2011 N/A

- P5 primer (HPLC purified): AATGATACGGACAGCACCAGATCACACTTCTTCCCTACACGGACGTCTTTCCGATCT
  - Konig et al., 2011 N/A

**Recombinant DNA**

- pPFMW (Gateway destination Vector for Drosophila expression)
  - The Drosophila Gateway Vector collection
  - https://emb.carnegiescience.edu/drosophila-gateway-vector-collection

- pPFHW (Gateway destination Vector for Drosophila expression)
  - The Drosophila Gateway Vector collection
  - https://emb.carnegiescience.edu/drosophila-gateway-vector-collection

- pPMW (Gateway destination Vector for Drosophila expression)
  - The Drosophila Gateway Vector collection
  - https://emb.carnegiescience.edu/drosophila-gateway-vector-collection

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LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Anne Ephrussi (ephrussi@embl.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All Drosophila melanogaster stocks were maintained at 25 °C, and throughout the study, samples were prepared from a mix of male and female flies. For the generation of transgenic flies from Gateway destination vectors, pUASP-based destination plasmids containing tagged EJC subunits were injected into fly embryos together with helper plasmid as described previously (Hachet and Ephrussi, 2004). To establish a control line for ubiquitous expression of epitope-tagged fusion proteins, the transgenic fly line w-; P[UASp-FLAG::MYC::EJC]/CyO; Sb/TM3 Ser1 was crossed with the driver line y1w*; If/CyO; P(w[+mC] = tub-P-GAL4)LL7/TM3, Sb1 Ser1 (Bloomington Stock 5138). To compare the degree incorporation of transgenic GFP-Mago, Myc-Y14 and FLAG-HA-eIF4AIII into endogenous EJCs, a fly line carrying all three transgenic EJC subunits and a transgene for ubiquitously expressed GAL4 was established by standard genetic crosses.

Briefly, the driver line y1w*; If/CyO; P(w[+mC] = tub-P-GAL4)LL7/TM3, Sb1 Ser1 (Bloomington Stock 5138) was crossed with the established transgenic line Myc-Y14 w*/If/CyO; P(UASp-MYC::Y14)/TM3 Ser1. Upon recombination and balancing of Myc-Y14 and the GAL4 driver transgene on the 3rd chromosome, the ubiquitously expressing Myc-Y14 fly line w*/If/CyO; UASp-MYC::Y14, P(w[+mC] = tub-P-GAL4)LL7/TM3 Ser1 was crossed with w*/P(UASp-FLAG::HA::eIF4AIII)/CyO; Sb/TM3 Ser1 and bw, mago-GFP::Mago;If/Cyo;Sb/TM3Ser (Newmark et al., 1997) to obtain the stable line bw-,mago-GFP::Mago;UASp-FlagHA::eIF4AIII/CyO;UASp-MYC::Y14, P(w[+mC] = tub-P-GAL4)LL7/TM3 Ser1, in which Myc-Y14, eIF4AIII were expressed under control of the UAS-GAL4 expression system and GFP-Mago under control of its own promoter. For sucrose density gradients, mRBP footprinting, and EJC ipaRt experiments, male and female flies of the following genotypes were used: w1118 (wild-type), bw-,mago-GFP::Mago;If/Cyo;Sb/TM3Ser expressing GFP-Mago under control of the mago promoter (Newmark et al., 1997), and the GFP tag-only expressing fly line y1w*, P(w[+mC] = act5C-GAL4)25F01/CyO, P(w[+mW.hs] = ubi-GFP.S65T)PAD1.
Fly stocks were amplified and two day-old flies were sedated by CO₂, quick-frozen and pulverized with a mortar under liquid nitrogen. Preparation of cytoplasmic lysates tagging of eIF4AIII, the destination vectors pTWF, pTWG and pPWF, pPWG were utilized in the recombination reaction. For N-terminal FLAG tagging, and pPMW for N-terminal MYC tagging of Y14 and eIF4AIII, respectively. For C-terminal FLAG and GFP tagging of elf4AII, the destination vectors pTWF, pTWG and pPW, pPWG were utilized in the recombination reaction.

Preparation of cytoplasmic lysates
Fly stocks were amplified and two-day-old flies were sedated by CO₂, quick-frozen and pulverized with a mortar under liquid nitrogen. Fly powder was taken up in lysis buffer [20mM HEPES pH8.0, 125mM KCl, 4mM MgCl₂, 0.1% IGEPA (Sigma-Aldrich), 1 Unit/ml Ribolock (Fermentas) and 1x CompleteMini Protease Inhibitor Cocktail (Roche)] in a 1:8 weight:volume ratio and subsequently transferred to glass tissue grinder (Kontes Glass, USA). All subsequent steps were carried out on ice. During a first round of grinding, a loose bulb pestle (Kontes Glass, USA) was utilized until a homogenate of fly tissues and lysis buffer was obtained. For the second round of grinding, a tight pestle (Kontes Glass, USA) was utilized to ensure sufficient rupturing of the cells. Nuclei and crude cytoplasm were separated by centrifugation for 10 min at 900 g at 4 °C. Nuclei were washed once in lysis buffer and quick-frozen for later use. For initial poly-A tail containing mRNA-RBP precipitation assay crude cytoplasmic supernatant was either left untreated or supplemented with DSP (Thermo Scientific) to 1mM final concentration and incubated for 1h at 4 °C with constant shaking. Upon completion of the DSP cross-linking reaction, both the treated and untreated crude cytoplasmic fractions were blocked with 25mM Tris-HCl pH7.5 and centrifuged at 25000 g for 30 min at 4 °C. Clarified cytoplasmic lysates were quick-frozen and stored at −80 °C for later use.

Cellular fractionation quality control
Cellular lysates obtained from “wild-type” w¹¹¹B flies were subjected to western blot analysis (see western blot analysis and antibody section). Western results of the separated cellular fractions confirmed the reliability of the assay (Figure S1A). The cytoskeletal motor protein Kinesin heavy chain (Khc) was present in cytoplasmic and absent from nuclear fractions; chromatin components such as histone 3 (H3) was detected primarily in nuclear and was nearly absent from cytoplasmic fractions (Figure S1B). Finally, known shuttling proteins such as the EJC subunits elf4AII and Y14 were present in both cellular fractions (Figure S1B).

Precipitation of mRNA-protein complexes
All procedures were carried out at 4 °C or on ice, except for the final elution step. To precipitate proteins in complex with poly-A containing mRNAs (mRNP), 15m of DSP treated and untreated (native) cytoplasm were evenly distributed in Petri dishes and exposed to UV radiation (254 nm) using a UV Crosslinker (Stratalinker) at an energy setting of 2x 150mJ/cm². UV irradiated and unexposed containing mRNPs, 15ml of DSP treated and untreated (native) were complemented with LCl, for a final salt concentration of 150mM KCl and 500mM LiCl. For mRNP precipitation, 100 μl equilibrated bead slurry of oligo-d(T)₂₅ Magnetic Beads (NEB) was mixed with 15ml salt-adjusted cytoplasmic lysate. Hybridization of poly-A containing mRNPs with oligo d(T)₂₅ beads was allowed to proceed for 2h, at 4 °C. Nucleotide mediated precipitation of mRNPs was stopped by pelleting oligo d(T)₂₅ beads 2 min on a magnet. Pelleted beads were mixed with 15ml high salt wash buffer [20mM HEPES pH 7.8, 750mM LiCl, 0.2% IGEPA(C) CA-630, 0.1% LiDS, 1mM DTT, 5 mM EDTA, 1x CompleteMini Protease Inhibitor Cocktail (Roche)] and incubated for 30 min at 4 °C. Washed beads were re-suspended in 1.5ml high salt wash buffer and washing was repeated two additional rounds for 10 min at 4 °C. After high salt washing was completed, beads were re-suspended in medium salt washing buffer [20mM HEPES pH 7.8, 0.5M LiCl, 0.2% IGEPA(C) CA-630, 0.1% LiDS, 1mM DTT, 5 mM EDTA, 1x CompleteMini Protease Inhibitor Cocktail (Roche)] and incubated for 30 min at 4 °C. Washed beads were re-suspended in 1.5ml high salt wash buffer and washing was repeated two additional rounds for 10 min at 4 °C. After high salt washing was completed, beads were re-suspended in medium salt washing buffer [20mM HEPES pH 7.8, 0.5M LiCl, 0.2% IGEPA(C) CA-630, 0.1% LiDS, 1mM DTT, 5 mM EDTA, 1x CompleteMini Protease Inhibitor Cocktail (Roche)] and incubated for 30 min at 4 °C. Washed beads were re-suspended in medium salt washing buffer. To minimize contamination by unrelated rRNPs or other RNA-protein complexes, two additional washing steps were performed in 1.5ml low salt LiDS buffer [20mM HEPES pH 7.8, 250mM LiCl, 0.2% IGEPA(C) CA-630, 0.05% LiDS 1mM DTT, 5 mM EDTA, 1x CompleteMini Protease Inhibitor Cocktail (Roche)] and washed in one subsequent washing step in 1.5ml low salt buffer without LiDS [20mM HEPES pH 7.8, 250mM LiCl, 0.2% IGEPA(C) CA-630, 1mM DTT, 5mM EDTA, 1x CompleteMini Protease Inhibitor Cocktail (Roche)]. For elution of mRNPs, the hybridized to oligo d(T)₂₅ magnetic beads were mixed with 20μl HE elution buffer [20mM HEPES pH8.0, 1mM EDTA] and incubated for 5 min at 30 °C, with agitation at 1200rpm. Elution was repeated two additional rounds. Supernatants were pooled and stored on ice for later use.

METHOD DETAILS

Topo and Gateway cloning
For entry clone preparation, the full-length Y14 coding region was PCR amplified from Drosophila melanogaster cDNAs using caccATGGCCGATG-TGTTGGACATTG3' as the forward primer and 5'TCGCGACGCTTTT-GGAACGTG3' as the reverse primer. Similarly, the eGFP full-length coding region was PCR amplified from plasmid pPWG (see below, destination vector recombination) using caccATGGTGAGCAAG-GGC'3 as the forward primer and 5'TCTTGTACGCTGTCATGC3' as the reverse primer. Directional Topo cloning into pENTR/SD/D-TOPO plasmid (Thermo Fisher) was performed according the manufacturer’s protocol. Preparation of an entry clone for the full-length elf4AII coding region was described elsewhere (Ghosh et al., 2014).

Recombination of entry clones with destination vectors from the Drosophila Gateway vector collection (gift of Terence Murphy, Carnegie Institution for Science; see link https://emb.carnegiescience.edu/) was performed according the Gateway cloning protocol (Thermo Fisher). Y14, elf4AII and eGFP entry clones were used for recombination with the destination vectors pPFMW and pPFHW for N-terminal FLAG tagging, and pPMW for N-terminal MYC tagging of Y14 and elf4AII, respectively. For C-terminal FLAG and GFP tagging of elf4AII, the destination vectors pTWF, pTWG and pPW, pPWG were utilized in the recombination reaction.
**Immunoprecipitation and RNase treatment**

All procedures were carried out at 4 °C or on ice. Whenever indicated, cytoplasmic extracts utilized were either untreated (native) or treated with DSP. Herein, DSP was directly added to the cell-homogenate prior to nuclear-cytoplasmic fractionation. DSP cross-linking was allowed in total for 1h, during cell factionation and cytoplasmic clarification step, and finalized by addition of 25mM Tris-HCl pH7.5. For pre-clearing of individual immunoprecipitation (IP) reactions, 10-15ml cytoplasmic lysate (4-5mg/ml protein-conc.) was incubated 1h at 4 °C with 150μl slurry of Protein A/G agarose-beads (Roche) to reduce resin mediated stickiness in all subsequent steps. After pre-clearing was completed, beads were depleted by centrifugation for 10min at 900 g and 4 °C. The reaction supernatant was transferred to fresh 15ml falcon tubes and stored on ice for immediate use. Prior to use, 20μl IP beads specific for the Flag or Myc epitope (Sigma) or 10μl GFP-trap beads (Chromotek) were incubated for 1h at 4 °C in lysis buffer containing 1% western blocking reagent (Roche) and 0.1mg/ml heparin (Sigma-Aldrich). Blocked IP beads were collected by centrifugation 5 min at 900 g, 4 °C prior their use in subsequent IP reactions. IP reactions on pre-cleared lysates was allowed to proceed for 3-4h at 4 °C with continuous agitation. Reactions were stopped by 5 min centrifugation at 900 g, 4 °C.

For washing of IP beads with standard stringency, collected beads from IP reaction were resuspended in 1.5ml ice cold high salt wash buffer [20mM HEPES pH8.0, 400mM KCl, 1mM MgCl2, 0.2% IGEPAL CA-630, 1mM DTT, 0.1 Unit/ml Riboloc (Fermentas) and 1x CompleteMini Protease Inhibitor Cocktail (Roche)]. Beads were washed for 10min at 4 °C with continuous agitation. Washed beads were collected by centrifugation at 4 °C, 900 g for 5min, resuspended high salt wash buffer and subjected to two additional rounds of high salt washing steps. The washed beads were transferred to fresh tubes and mixed with 1.5ml low salt wash buffer [20mM HEPES pH8.0, 250mM KCl, 1mM MgCl2, 0.2% IGEPAL CA-630, 1mM DTT, 0.1 Unit/ml Riboloc (Fermentas) and 1x CompleteMini Protease Inhibitor Cocktail (Roche)]. Subsequent washing steps were executed as for high salt washing. To further minimize risk of protein contaminants, washed beads were transferred to new reactions tubes, rinsed with ice-cold 1ml PBS and placed on ice for later use.

For washing of IP beads with high stringency, individual wash was performed for 15 min. Three series of IP bead washes were applied using high salt wash buffer [20mM HEPES pH 7.8, 750mM NaCl, 0.1% IGEPAL CA-630, 0.1% Na-deoxycholate, 0.1% SDS, 1mM DTT, 0.02mg/ml Heparin, 1x CompleteMini Protease Inhibitor Cocktail (Roche)], medium salt wash buffer [20mM HEPES pH 7.8, 250mM NaCl, 0.1% IGEPAL CA-630, 0.1% Na-deoxycholate, 0.02% SDS, 1mM DTT, 0.02mg/ml Heparin, 1x CompleteMini Protease Inhibitor Cocktail (Roche)] and low salt wash buffer [20mM HEPES pH 7.8, 150mM NaCl, 0.02% IGEPAL CA-630, 0.01% Na-deoxycholate, 1mM DTT, 0.02mg/ml heparin, 1x CompleteMini Protease Inhibitor Cocktail (Roche)]. Herein for each washing series four consecutive washing steps were executed. Upon completion of each washing series, the IP beads were transferred into new clean reaction tubes. Finally, as with standard stringency washings, the washed beads were transferred to new reactions tubes, rinsed with ice cold 1ml PBS and placed on ice for later use. To distinguish direct protein to protein interactions from interactions mediated by RNA templates, beads from the IP reaction were resuspended in 15ml PBS before the washing was performed and supplemented with 100 units of RNaseI (Thermo Fisher). Initial round of RNA fragmentation was allowed for 1h at 4 °C under permanent agitation. Subsequent washing was performed under highly stringent washing conditions as described above. After finalization of the last washing step IP beads were resuspended in 1ml PBS and completed with 0.2 units of RNaseI. Final RNA fragmentation was incubated for 5 min at 37 °C, with agitation at 1300rpm. The reaction was stopped on ice and IP beads were subjected to an additional round of high stringency washing. After the final wash, the IP beads were rinsed with ice cold 1ml PBS, transferred into fresh tubes and stored on ice for later use.

**Choice of EJC bait: GFP-Mago**

To test which epitope-tagged EJC subunit, would serve as the optimal IP bait, we established a fly line in which transgenes encoding GFP-Mago, MYC-Y14 and FLAG-HA-eIF4AIII were co-expressed (see Key Resources Table). Expression of GFP-Mago was under control of the mago promoter, while the expression of the other transgenes was driven globally by UAS-GAL4 system (Duffy, 2002; Rorh, 1998). To monitor any treatment-specific effects, experiments were executed simultaneously on dithio(bis)-succinmimidyl propanoate (DSP) (cross-linked (Lomant and Fairbanks, 1976) and native cytoplasmic lysates.

Results obtained from western blotting (Figure S1B) suggested that DSP treatment did not lead to unspecific protein stickiness, as judged from the lack of signal for any of the proteins probed in the control IPs (Figure S1B; lanes 3 and 8). Further confirming stringency of the assay, the non-RNA binding Khc was exclusively detected in lysate “inputs” but not in any of the SDS-PAGE resolved precipitates (Figure S1B; lanes 1, 3-6, 8-11). Independent of the conditions tested, in anti-MYC precipitates we observed strong signals of MYC-Y14 itself and of transgenic GFP-Mago, whereas endogenous elf4AIII and its transgenic FLAG-HA tagged counterpart were absent or only marginally detected (Figure S1B; lanes 4 and 9). Anti-FLAG precipitates specific for FLAG-HA-elf4AIII showed strong signals for the transgenic bait itself, but signals for its transgenic and endogenous interaction partners Y14 and Mago were weak when compared with the signals in the input lysate (Figure S1B; lanes 5 and 10). In our hands, the only transgenic bait that displayed sufficient incorporation into the EJC, was GFP-Mago: anti-GFP precipitates showed strong signals for all probed EJC subunits, including GFP-Mago, endogenous elf4AIII, Y14, as well as the transgenic MYC-Y14, anti-GFP precipitates also showed reproducibly weak signals for FLAG-HA-elf4AIII upon DSP cross-linking, but only to an extent similar to what was observed within anti-MYC IPs (Figure S1B; lanes 6 and 11). Interestingly in all IPs except the control we detected signals for the genuine mRNP component poly-A binding protein PABP. (Figure S1B; lanes 1, 3-6, 8-11). For GFP-specific IPs this was not surprising, due to the efficient incorporation of GFP-Mago into cytoplasmic EJCs. However for IPs in which FLAG-HA-elf4AIII served as bait and no other EJC subunits were detected, this result was not anticipated (Figure S1B; lanes 5 and 10), and questioned the restrictedness...
of eIF4AIII to EJC (see EJC Sedimentation). Conversely, the efficient co-IP of EJC subunits with GFP-Mago qualified to us GFP-Mago as the bait of choice for subsequent EJC specific IP assays.

Sucrose density gradient centrifugation
Pulverized flies were resuspended in lysis buffer supplemented with 0.4mM cycloheximide (Sigma-Aldrich). All subsequent steps for cellular fractionation were executed as described above. To resolve cytoplasm into fractions containing mRNPs, ribosomes or polysomes, typically 150µl of lysate were loaded on a 3.4ml 10%–50% linear sucrose gradient. Sucrose gradients were made using 20mM HEPES pH8.0, 150mM KCl, 4mM MgCl2, 0.4mM Cycloheximide, 0.0.5% IGEPA L CA-630, 0.2% Na-deoxycholate, 1 Unit/ml Ribolock (Fermentas), and 1x CompleteMini Protease Inhibitor (Roche) using a gradient mixer (Biconomp) and fraction collector (Bio-comp), following the manufacturer’s protocol. Protein-RNA complexes were separated by ultracentrifugation in a SW60Ti rotor (Beckman) at 50000rpm, at 4°C for 30 min. Fractions were collected batch-wise in 100µl aliquots. Nucleic acid content in the fractions was measured manually at 254nm. For protein analysis, fractions were supplemented with trichloracetic acid (30%) and kept on ice for 15 min to ensure protein precipitation. Protein pellets were subsequently washed with ice-cold acetone and denatured for 10 min at 95°C in 2x LDS sample buffer (Thermo Fisher), 10mM DTT.

EJC sedimentation: eIF4AIII is a poor bait
Since not only FLAG-HA (see Pilot IP) but also other tagged versions of eIF4AIII failed to incorporate efficiently into endogenous EJCs (data not shown), we wondered whether the RNA-binding DEAD-box helicase might also be associated with RNAs in an EJC-unrelated manner. We therefore compared sedimentation profiles of endogenous cytoplasmic Y14 and eIF4AIII obtained by ultracentrifugation of cytoplasmic lysates in sucrose density gradients, and monitored the content of nucleic acids by UV absorption and accompanying content of proteins by western blotting (Figure S1C). Supporting the notion that the EJC is a component of mRNPs, Y14 co-sedimented with eIF4AIII in light mRNP fractions (Figure S1C; lanes 2-5). Contradicting, however, the exclusive function eIF4AIII in EJC related processes, we also observed eIF4AIII (but not Y14) co-sedimenting with ribosomal subunit proteins such as RpS6 and RpL32 in the 40/48 s and 60 s fractions, and with high-density polysomes (Figure S1C, lanes 2-17, 28-30, 32). This unexpected result indicated to us a yet undefined function for Drosophila eIF4AIII outside of an EJC context. This for us disqualified the DEAD-box RNA helicase eIF4AIII as bait for EJC specific immunoprecipitations.

WESTERN ANALYSIS AND ANTIBODIES
Washed IP beads were mixed with 2x LDS sample buffer (Thermo Fisher) containing 50mM DTT (Sigma-Aldrich). Eluted mRNPs from oligo-d(T)25 precipitates samples as well as input samples were mixed in 1:1 ratio with 4X LDS sample buffer, 100mM DTT. To allow reduction of covalent DSP mediated bonds, the samples were incubated for 20min at 42°C (Beckman) at 50000rpm, at 4°C Ribolock (Fermentas), and 1x CompleteMini Protease Inhibitor (Roche) using a gradient mixer (Bio-comp) and fraction collector (Bio-comp), following the manufacturer’s protocol. Protein-RNA complexes were separated by ultracentrifugation in a SW60Ti rotor (Beckman) at 50000rpm, at 4°C for 30 min. Fractions were collected batch-wise in 100µl aliquots. Nucleic acid content in the fractions was measured manually at 254nm. For protein analysis, fractions were supplemented with trichloracetic acid (30%) and kept on ice for 15 min to ensure protein precipitation. Protein pellets were subsequently washed with ice-cold acetone and denatured for 10 min at 95°C in 2x LDS sample buffer (Thermo Fisher), 10mM DTT.

Mass spectrometry
For tandem mass spectrometry, immunoprecipitates were submitted for further preparation and analysis to the EMBL Proteomics Core facility. All reagents were prepared in 50 mM HEPES (pH 8.5). For reduction of cysteines, dithiothreitol was added (56°C, 30 minutes, 10 mM); further alkylation was performed using iodacetamide (10 mM, for 30 minutes in the dark, at room temperature). Samples were prepared for LC-MS/MS using the SP3 protocol (Hughes et al., 2014), digestion was performed using trypsin (1:50 enzyme:protein ratio) at 37°C overnight. TMT10plex Isobaric Labeling (ThermoFisher) was performed according the manufacturer’s instructions. The OASIS® HLB Elution Plate (Waters) was used for sample clean-up. Offline high pH reverse phase fractionation was performed as described previously (Reichel et al., 2016). In brief, the samples were run on an Agilent 1200 Infinity high-performance liquid chromatography (HPLC) system equipped with a Gemini C18 column (3 µm, 110 Å, 100 x 1.0 mm, Phenomenex). The solvent system consisted of 20 mM ammonium formate (pH 10.0) as mobile phase-A, and 100% acetonitrile as mobile phase-B. After offline fractionation, peptides were separated using the UltiMate 3000 RSLC nano LC system (Dionex) fitted with a trapping cartridge (µ-Precolumn C18 PepMap 100, 50 µm, 300 µm i.d. x 5 mm, 100 Å) and an analytical column (Acclaim PepMap 100 75 µm x 50 cm C18, 3 µm, 100 Å). The outlet of the analytical column was coupled directly to a QExactive plus (Thermo) using the proxen nanoflow source in positive ion mode. Solvent A was water, 0.1% formic acid and solvent B was acetonitrile, 0.1% formic acid. Trapping time was 6 minutes at a constant flow of solvent A at 30 µL/ min onto the trapping column. Peptides were eluted via the analytical column at a constant flow of 0.3 µL/ min. During elution, the percentage of solvent B increased in a linear fashion from 2% to 4% B in 4 minutes, from 4% to 8% in 2 minutes, then 8% to 28% for a further 96 minutes, and finally from 28% to 40% in another
10 minutes. Column cleaning at 80% B followed, lasting 3 minutes, before returning to initial conditions for the re-equilibration, lasting 10 minutes. The peptides were introduced into the mass spectrometer (QExactive plus, ThermoFisher) via a Pico-Tip Emitter 360 μm OD x 20 μm ID; 10 μm tip (New Objective) and a spray voltage of 2.3 kV was applied. The capillary temperature was set at 320 °C. Full scan MS spectra with mass range 350-1400 m/z were acquired in profile mode in the FT with resolution of 70,000. The filling time was set at maximum of 100 ms with a limitation of 3x10^6 ions. DDA was performed with the resolution of the Orbitrap set to 35000, with a fill time of 120 ms and a limitation of 2x10^5 ions. Normalized collision energy of 32 was used. A loop count of 10 with count 1 was used and a minimum AGC trigger of 2^3 was set. Dynamic exclusion time of 30 s was applied. The peptide match algorithm was set to ‘preferred’ and charge exclusion ‘unassigned’, charge states 1, 5 - 8 were excluded. Isolation window was set to 1.0 m/z and 100 m/z set as the fixed first mass. MS/MS data was acquired in profile mode.

EJC ipaRt and mRBP footprinting

For EJC ipaRt and mRBP footprinting assays, DSP was added directly to Lysisbuffer prior to preparation of the fly cell homogenate (compare with Cytoplasmic Lysate preparation). Crosslinking was allowed for 1h at 4 °C and stopped by addition of 25mM Tris-HCl pH7.5 prior clarification of cytoplasmic lysates. All steps for cDNA library preparation were adapted and modified from the previously published iCLIP protocol described by König and co-workers (Konig et al., 2011). Barcoded primer sequences for reverse transcription, cut-oligo sequence and cDNA amplification primers for Illumina sequencing are summarized in Table S3 in the section supplementary information.

EJC ipaRt and L3-App adaptor ligation

For isolation of protein (holo-)complexes associated with RNA templates (ipaRt), DSP treated cytoplasmic extracts were supplemented with 0.02% heparin. Subsequently, cytoplasmic fractions from GFP-Mago and GFP expressing flies were mixed with 100μl equilibrated bead slurry of protein A/G agarose (1:1 mixture) for pre-clearing. Pre-clearing proceeded for 1h at 4 °C with continuous agitation. GFP-trap beads (Chromotek) and Protein A/G (Roche) the “beads-only” control were blocked for 1h at 4 °C in 20mM HEPES pH7.8, 125mM KCl, 0.1% IGEPAL CA-630, 0.1mg/ml Heparin, 4mM MgCl2, 1x CompleteMini Protease Inhibitor Cocktail (Roche), 20 Units/ml Ribolock. Next, 15ml of heparin complemented and pre-cleared cytoplasmic lysate were mixed with 20μl immunoprecipitation resin. IP reactions were incubated for 4h at 4 °C with constant agitation. Washing buffer compositions in ipaRt were the same as those used for washing IPs under highly stringent conditions (see above). However, each complete wash consisted of a series of 5 repeated washes. To reduce the likelihood of transfer of nucleic acid contaminants, at the end of each a wash step the IP bead slurry was transferred into a clean new reaction tube. After the final washing step bead aliquots were transferred into clean new reaction tubes and adjusted to 1ml isonitric reaction buffer [20mM HEPES pH 7.8, 150mM KCl, 4mM MgCl2, 0.02% IGEPAL CA-630, 1x CompleteMini Protease Inhibitor Cocktail (Roche)] containing 1 Unit RNaseI and 4 Units TURBO DNaseI (Thermo Fisher). The RNase-DNase reaction was allowed to proceed for 5min at 37 °C with continuous agitation (1200rpm). To stop the RNase reaction, ipaRt bead aliquots were placed on ice, immediately rinsed with high salt wash buffer and transferred to new clean reaction tubes. Subsequently, ipaRt beads were subjected to two rounds of alternating high salt and low salt washing steps. After every individual washing step, the ipaRt beads were transferred into a clean new reaction tube. When washing was completed, beads were rinsed twice with 1ml isonitric reaction buffer [20mM HEPES pH 7.8, 150mM KCl, 4mM MgCl2, 0.02% IGEPAL CA-630, 1x CompleteMini Protease Inhibitor Cocktail (Roche)] and placed on ice for later use. For 3’ end de-phosphorylation of protected RNA fragments, ipaRt beads were taken up in 50 μl T4-PNK reaction mix [70mM Tris-HCl pH6.5, 10mM MgCl2, 5mM DTT, 10 Units T4-PNK (Fermentas), 20 Units Ribolock (Fermentas)]. De-phosphorylation proceeded for 45min at 37 °C and continuous shaking at 1100rpm. The T4 PNK reaction was stopped by placing the samples on ice. Immediately thereafter, the beads were exposed to two rounds of repetitive high salt and low salt washing steps (as above) and finally rinsed with ice-cold PBS. For the ligation of L3-App DNA adaptor (Konig et al., 2011) to co-immunoprecipitated 3’ end dephosphorylated RNA fragments, ipaRt beads were resuspended in 40μl T4 RNA ligase mix composed of 50mM Tris-HCl pH 7.5, 10mM MgCl2, 10mM DTT, 5% PEG400 (Sigma-Aldrich), 20 Units RNaseOUT (Sigma-Aldrich), 1.5μM L3-App adaptor and 10 Units T4 RNA Ligase 2 (truncated) (NEB). Ligation was allowed to proceed for 15h at 16 °C, with constant agitation at 1100rpm. To remove non-ligated L3-App DNA adaptor after ligation, ipaRt beads were subjected to 2x high salt, 2x medium salt and 2x low salt washing steps, with a transfer of the beads into new clean reaction tubes every second wash. To extract the L3-App-RNA ligation products, ipaRt beads were mixed with 200μl proteinase K reaction buffer [100mM Tris-Cl pH 7.4; 50mM NaCl; 10mM EDTA] complemented with 200μg proteinase K (Fermentas). The proteolysis reaction was incubated for 20min at 37 °C, 1100rpm, then supplemented with 200μl proteinase K urea reaction buffer [100mM Tris-HCl pH 7.4, 50mM NaCl, 10mM EDTA, 6M urea] and incubated for an additional 45min at 37 °C, with constant shaking at1100rpm. For extraction of RNA-DNA ligation products, the reaction samples were vortexed 5 min with 40μl phenol/chloroform/isoamylalcohol (25:24:1) following standard DNA extraction protocols. RNA-DNA ligation products were buffered with sodium acetate pH5.2 and finally subjected to ethanol precipitation at −20 °C over-night using 5μg linear acrylamide (Thermo Fisher) as carrier.

mRBP-footprinting and L3-App adaptor ligation

mRBP footpring on poly-A tail precipitated mRNPs (see above) was performed using RNaselo. To do so, mRNP elution aliquots were mixed and adjusted to 1ml isonitric buffer [20mM HEPES pH 7.8, 150mM KCl, 4mM MgCl2, 0.02% IGEPAL CA-630, 1x CompleteMini Protease Inhibitor Cocktail (Roche)] containing 1 Unit RNasel (NEB) and 4 Units TurboDNasel (Thermo Fisher).
RNase-DNase digestion was allowed to proceed for 5 min at 37 °C with constant agitation (1200 rpm). RNA digestion was stopped by placing samples on ice and adding 0.5% LIDS. To separate short RNA digestion products from mRNA fragments in complex with RBPs, samples were placed into an Amicon Ultra 10K concentrator columns (cutoff 10kDa, Millipore) and centrifuged at 4 °C for a final volume of 50μl according to the manufacturer’s instructions. The resulting concentrates were dissolved in 10 volumes of HE buffer containing 0.5% LIDS and subjected to a second round of concentration. Finally, 50μl MRBP-RNA concentrates were supplemented with 5μl 1M DTT, incubated 20 min at 42 °C to reverse the DSP mediated protein-protein cross-linking. Upon reduction of covalent bonds, samples were supplemented with 200μl proteinase K reaction buffer [100mM Tris-HCl pH 7.4, 50mM NaCl, 10mM EDTA, 0.2% SDS], 10μl proteinase K (20mg/ml, Fermentas) and incubated for 20 min at 37 °C. Next, 200μl proteinase K reaction buffer [100mM Tris-HCl pH 7.4, 50mM NaCl, 10mM EDTA, 6M urea, 0.2% SDS] was added and protein digestion was allowed to proceed for additional 40 min at 37 °C. To stop protein digestion, samples were diluted with 3 volumes of Trizol LS (Thermo Fisher). With the exception of the overnight RNA precipitation at −20 °C, all steps to isolate RBP protected mRNA fragments were performed according instructions in the Trizol LS manual (Thermo Fisher). For removal of 3’ phosphorylated ends, after RNase digestion RNA the pellets were resuspended directly in 50 μl ddH₂O, and adjusted to a final volume of 100μl with 70mM Tris HCl pH6.5, 10mM MgCl₂, 5mM DTT, 10 Units T4-PNK (Fermentas), 20 Units Ribolock (Fermentas). The PKN reaction was allowed to proceed for 45 min at 37 °C, with agitation at 1100 rpm and stopped by addition of 3 volumes of Trizol LS (Thermo Fisher). As described above, all steps for recovery of RNA except for overnight RNA precipitation at −20 °C were performed following the instructions in the Trizol LS manual (Thermo Fisher). Pellets of de-phosphorylated and cleaned RNA fragments were resuspended in 20μl ddH₂O. For ligation of the L3-App adaptor (Konig et al., 2011, see oligonucleotide list in Supplementary Information), RNA samples were adjusted to a 40μl ligation reaction composed of 50mM Tris-HCl pH 7.5, 10mM MgCl₂, 10mM DTT, 5% PEG4000 (Sigma-Aldrich), 20 Units RNaseOUT (Sigma-Aldrich), 1.5μM L3-App adaptor and 10 units of T4 RNA Ligase 2 (truncated) (NEB). Ligation proceeded for 15 min at 16 °C with constant agitation (1100rpm). To reduce the content of non-ligated L3-App adaptor in the samples after completion of the ligation reaction, aliquots were mixed with 1ml HE elution buffer [20mM HEPES pH8.0, 1mM EDTA] and concentrated in Amicon Ultra 10K concentrator columns until a volume of 100μl was reached. RNA-DNA ligation products were extracted with a mix of phenol, chloroform and isomyl alcohol (25:24:1) mixed with 0.1 volumes 3M Na-Acetate pH 5.2, 5μg linear acrylamide (Thermo Fisher) and finally precipitated in 2 volumes ethanol over night at −20 °C.

### cDNA library preparation

Barcoded primer sequences for reverse transcription, cut-oligo sequence and cDNA amplification primers for Illumina sequencing are listed in Supplementary Information and in the published iCLIP protocol by König and co-workers (Konig et al., 2011). First, pellets of RNA-L3-App products were washed in 80% ice-cold ethanol and subsequently resuspended in 10μl ddH₂O. For reverse-transcription, the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen) was utilized. Herein 8μl of sample solution were mixed with 1μl solution of RTclip-Primer mixture (each 0.5 μM) and 1μl Annealing cocktail (Invitrogen). To anneal the RTclip primer with complementary sites in the L3-App adaptor sequence, sample-primer mixture aliquots were denatured for 3 min at 70 °C and allowed to anneal at 25 °C for 10 min. Subsequently, samples were supplemented with 10μl 2x Reaction Mix (Invitrogen) and 2μl Superscript III/RNaseOUT™ enzyme mix (Invitrogen). Reverse transcription was performed in three steps using a C1000 Touch Thermal Cycler (Biorad): 1st for 20 min at 85 °C, followed by 23-26 amplification cycles. Each cycle comprised 1 s denaturation at 90 °C. To avoid re-hybridization of RNA and cDNA, reverse transcribed samples were immediately placed on ice, topped up to a volume of 100μl with ice-cold ddH₂O, and supplemented with 500μl guanidine-HCl-containing PB buffer from the PCR purification kit (QIAGEN). Samples were thereafter transferred into QIAquick columns (QIAGEN) and washed according to the manufacturer’s instructions. Elution was performed in two consecutive steps using at each step 10μl warm (60 °C) elution buffer (QIAGEN). For circularization of the cDNA products, purified samples were adjusted to a 40μl final reaction volume composed of 1X CircLigase Buffer II (Epicenter), 500μM MnCl₂, 60 Units CircLigase II (Epicenter). cDNA circularization proceeded for 1h at 60 °C. After circularization, the samples were mixed with 11μl ddH₂O, 4.5μl 10x restriction Buffer 4 (NEB), 1.5μl Cut-Oligo [10μM, see table] and incubated in a C1000 Touch Thermal Cycler (Biorad) applying a program comprising an initial 4min, 85 °C denaturation followed by sequential cooling to 25 °C in 1 °C steps every 10 s. After hybridization, the samples were completed with 3 μL BamHI HF (NEB) for digestion of Cut-Oligo RTclip primer hybridization sites. Linearization was carried out for 30min at 37 °C. Linearized barcoded cDNAs were subjected for extraction to the QIAquick PCR purification protocol (QIAGEN). cDNA elution was performed in two consecutive steps using at each step 14μl warm (60 °C) elution buffer (QIAGEN). For cDNA amplification 14μl eluted sample was mixed with an Illumina sequencing compatible P3/P5 primer mixture [1μM final concentration each] and 1x Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher) to a final volume of 20μl. Amplification was performed in a C1000 Touch Thermal Cycler (Biorad). Initial denaturation was performed for 20 s at 98 °C, followed by 23-26 amplification cycles. Each cycle comprised 1 s denaturation at 98 °C, 10 s annealing at 65 °C and 15 s elongation at 72 °C. The final elongation step was extended to 1 min. PCR products were desalted and cleaned using the QIAquick PCR purification protocol (QIAGEN). Purified PCR products were resolved by standard gel-electrophoresis in 1x TBE, 4% MetaPhor Agarose (Lonza) gels at 4 °C. To obtain an optimal insertion size and exclude primer duplicates, cDNA amplicons migrating between 200 and 300 bp were excised and extracted by MinElute Gel Extraction kit (QIAGEN). Depending on the yield, 5-12ng of purified cDNA library were obtained and submitted to the EMBL GeneCore Facility. Strand specific libraries were sequenced single-end with 55 bp on an Illumina HiSeq2000.
mRNA Sequencing

Total mRNA was extracted from whole flies using Trizol LS (Thermo Fisher) according to the manufacturer’s instructions. 10µg of purified total RNA were depleted of rRNA and small RNAs through two consecutive poly-A mRNA capture steps, using oligo-dT25 coated magnetic Dynabeads (Sigma-Aldrich) according to the manufacturer’s protocol. 500ng purified mRNA were submitted for library preparation. Barcoded stranded mRNA-seq libraries were prepared using the Illumina TruSeq RNA Sample Preparation v2 Kit (Illumina, San Diego, CA, USA) implemented on the liquid handling robot Beckman FXP2. Obtained libraries were pooled in equimolar amounts; 1.8 pM solution of this pool was loaded on an Illumina NextSeq 500 sequencer and sequenced bi-directionally, generating ~500 million of paired reads, each 85 bases long.

Computational data processing

Computational analysis was executed in R (R Core Team, 2017). Representative plots were generated with Microsoft Excel or with ggplot2 (Wickham, 2009) package in R. For individual R packages see Key Resources Table and Results section.

PROCESSING OF MASS SPECTROMETRY DATA

Acquired data were processed by IsobarQuant (Franken et al., 2015) and Mascot (v2.2.07), searched against a Uniprot Drosophila melanogaster proteome database (UP000000803) containing common contaminants and reversed sequences. The data were searched with the following modifications: Carbamidomethyl (C) and TMT10 (K) (fixed modification), Acetyl (N-term), Oxidation (M) and TMT10 (N-term) (variable modifications). The mass error tolerance for the full scan MS spectra was set to 10 ppm and for the MS/MS spectra to 0.02 Da. A maximum of 2 missed cleavages was allowed. For protein identification a minimum of 2 unique peptides with a peptide length of at least seven amino acids and a false discovery rate below 0.01 were required on the peptide and protein level. To define relative enrichment of candidates in GFP-Mago precipitates versus GFP-control, detected proteins were subjected to expression set analysis by Limma (Ritchie et al., 2015). Limma results, including enrichment, significance and adjusted p value, are summarized in the Table S4: Limma Results from TMT labeled mass spectrometry.

Sequencing read mapping

mRBP footprinting and ipaRt reads were demultiplexed using the sample barcode and the unique molecular barcode was appended to the read name in the fastq file. These reads are then trimmed using fqtrim (https://ccb.jhu.edu/software/fqtrim/) with the adaptor sequence AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTATGCCGTCTTCTGCTTG. Trimmed reads shorter than 20bp were discarded. The ipaRt, mRBP-footprinting and RNA-seq reads from Illumina deep sequencing were mapped to the Drosophila melanogaster reference genome (dm6, gtf from BGDP version 81) using the transcriptome alignment option in TopHat2 (Kim et al., 2013). Only uniquely mapped reads were used, and in the ipaRt and mRBP-footprinting samples duplicates were removed using the random barcode at the start of the forward read. For analysis of human iCLIP and mRNA-seq data from Hauer et al. (2016), reads from GEO accession GEO: E-MTAB-4215 were processed the same way, using hg38 and gtf annotation 82 from ensembl.

Assay quality control: gene class enrichment

Stabilization of protein-protein interactions by DSP cross-linking in ipaRt and mRBP footprinting (Figure S1D) might result in the trapping of unwanted proteins and RNPs such as ribosomes (and RNAs within) within larger mRNP composite complexes. Although we observed a general reduction of EJC unrelated proteins in GFP-Mago precipitates upon RNaseI fragmentation (see main result section and Figure S2A), we found that approximately 8% of all sequencing reads from EJC ipaRt and mRBP footprinting were mapping non-uniquely in the Drosophila genome (Figure S2B) to intergenic, intronic, and non-coding RNA sites (Figure S2C). This suggests that, to a certain extent, mRNP-unrelated RNP complexes were co-precipitated in both EJC ipaRt and mRBP footprinting assays.

To test whether the minor abundance of reads mapping at multiple site in the genome was due to DSP cross-linking, we analyzed sequencing results from EJC ipaRt, mRBP footprinting and mRNA-Seq (Figure S4A) for gene-class enrichment by expression set analysis using DESeq (Anders and Huber, 2010; Love et al., 2014). DESeq analysis of EJC ipaRt and mRNA-Seq expression analysis revealed an EJC enrichment for non-coding transcripts such as rRNAs, tRNAs, snRNAs, while the protein-coding and non-coding gene classes transcribed by RNA Pol II appeared either unaffected or depleted, as was the case of single exon-genes (Figure S4B). Just as with EJC ipaRt, the DESeq analysis of sequencing results from mRBP-footprintning and mRNA-Seq showed an mRBP enrichment bias for rRNA and small non-coding RNAs (Figure S4C). Nevertheless, when we defined gene class enrichment by direct comparison of EJC ipaRt with mRBP footprinting, both of which were treated with DSP, primarily mRNAs of multi-exon protein coding genes showed a bias for EJC enrichment, while mRNAs of single-exon genes and genes of non-RNA Pol II transcripts were either unaffected or depleted (Figure S4D).

These findings suggest that for reduction of false positive estimates in RNA immunoprecipitation assays in which protein-protein crosslinking is implemented, a direct comparison of specific IP libraries with mRBP protected fragments is more appropriate than comparing with mRNA-Seq libraries. Given the strong enrichment of protein coding multi-exon genes in the EJC associated versus mRBP protected RNA fragments, we focused all subsequent analyses on this class of genes.
For assessing how different features explain EJC binding at the RNA level, a linear model was fitted, with the log 2-fold change (estimated from DESeq) between the isoforms of EJC ipaRt and mRBP footprinting regressed against number of introns, maximum intron length of transcript, transcript abundance, mRNA length and degree of alternative splicing. These features were estimated from RNAseq data (see Gene feature annotation section) and relative importance of each feature was estimated using the R relaimpo package (Groemping, 2006).

For assessing different features that explain EJC binding at the junction level, a linear model was fitted, with the delta log 2-fold change (estimated from DESeq between junction and gene EJC enrichment) regressed against splicing related features (see Splicing analysis and ISE, ESE, ESS), folding related features (see Prediction of RNA structures and transformation using Gaussian Mixture Model) and counts of the hexamers we identified. All features were scaled and centered before linear regression using lm() function in R.

RNA structure prediction and clustering
RNA structure prediction was performed using Vienna RNA Package (Lorenz et al., 2011). For each exon-exon junction in Drosophila, the 37nt upstream of the junction and the 28nt downstream of the junction were merged. Secondary structure of this 65nt long sequence was then predicted by using the following command line: “RNAplfold -T 21 -W 66 -u 1,” which assumes folding at 21°C. We obtained the pairing probability of each base pair and performed logit transformation on these probabilities. To reduce dimension on this bpp data, we fitted a Gaussian Mixture Model assuming equal variance of the clusters (“EII” model). The probabilities of the junctions falling into each category would be used in subsequent analysis as a variable for predicting junction EJC deposition. We tried 2 to 6 clusters, at each try regressing the Δlog 2-fold change against these probabilities. At 4 clusters, we observed no substantial increase in R² and used this number of clusters for subsequent analysis.

EJC protection site peak calling
Replicates were combined and the genomic coverage was calculated for the ipaRt samples. The genomic coverage was translated onto the transcriptome coordinates using GenomicFeatures (Lawrence et al., 2013) in R. We considered only transcripts that have a TPKM > 1. For each transcript, regions with more than 2x the mean coverage of the transcript were identified, and the maxima position called out for each of these regions, and defined as peak position. To estimate the log 2-fold change of a peak coordinate in ipaRt over mRBP footprinting, we took the surrounding 20nt (± 10nt) of each peak position, and calculate the log 2-fold change in read counts across all replicates. For final consideration of ipaRt peaks, we retained only peaks with a coverage of at least 30 and log 2-fold change over mRBP > 1.

DIFFERENTIAL EXPRESSION SET ANALYSIS

Genes expression was estimated by counting reads that overlapped with all annotated genes in BGDP version 81 gtf, using the countOverlaps function in GenomicAlignments. DESeq2 (Love et al., 2014) was used for estimation of log 2-fold change between ipaRt and mRBP footprinting or total mRNA. Samples were normalized using total library size for analysis across all genes (Figures S4B–S4D). For assessing EJC enrichment in protein coding genes, the default median normalization in DESeq2 was used. To estimate EJC enrichment at exon-exon junctions, reads that overlapped within 50bp upstream and 50bp downstream of exon-exon junction were counted similarly using the countOverlaps function, and DESeq2 was used on this count table.

Gene feature annotation
For each gene, we estimated the most abundant transcript using Kallisto (Bray et al., 2016) and used that as a representative of the gene. From this representative transcription, we calculated the number of introns, transcript length, maximum intron length of the gene. The transcript abundance of the gene was tpkm of the most abundant transcript. The Shannon entropy of each gene was calculated using the transcript abundance estimate obtained from Kallisto.

Splicing analysis and ISE, ESE, ESS
Prediction of 5’ splice strength was performed using MaxEntScan, (Yeo and Burge, 2004). Annotation of junctions with respect to ISE, ESE and ESS was done using hexamers provided from Brooks et al. (2011) and Wang et al. (2004).

Assessing features that explain EJC binding
For assessing how different features explain EJC binding at the RNA level, a linear model was fitted, with the log 2-fold change (estimated from DESeq) between ipaRt and mRBP footprinting regressed against number of introns, maximum intron length of transcript, transcript abundance, mRNA length and degree of alternative splicing. These features were estimated from RNAseq data (see Gene feature annotation section) and relative importance of each feature was estimated using the R relaimpo package (Groemping, 2006).

Sequencing read coverage across exon junctions
For exons of at least 100nt, coverage measurement within 50nt upstream and 50nt downstream of exon-exon junction was calculated from each bam file using coverage and Views function of the GenomicAlignments package (Lawrence et al., 2013) in R, while for exons of > 200nt length, coverage measurement within 100nt upstream and downstream of exon-exon junction was calculated. This per nt coverage was utilized for definition of coverage median coordinates within exon-exon junctions in EJC ipaRt and mRBP footprinting assays. When a 5’ splice site was observed to undergo splicing reaction with 2 alternate 3’ splice sites, we assigned coverage within 5’ exons to corresponding exon-exon junction isoforms based on the ratio of reads that span either junction. The reciprocal approach was executed when a 3’ splice site was observed to undergo splicing with alternate 5’ splice sites.

RNA structure prediction and clustering
RNA structure prediction was performed using Vienna RNA Package (Lorenz et al., 2011). For each exon-exon junction in Drosophila, the 37nt upstream of the junction and the 28nt downstream of the junction were merged. Secondary structure of this 65nt long sequence was then predicted by using the following command line: “RNAplfold -T 21 -W 66 -u 1,” which assumes folding at 21°C. We obtained the pairing probability of each base pair and performed logit transformation on these probabilities. To reduce dimension on this bpp data, we fitted a Gaussian Mixture Model assuming equal variance of the clusters (“EII” model). The probabilities of the junctions falling into each category would be used in subsequent analysis as a variable for predicting junction EJC deposition. We tried 2 to 6 clusters, at each try regressing the Δlog 2-fold change against these probabilities. At 4 clusters, we observed no substantial increase in R² and used this number of clusters for subsequent analysis.
Tree model for mRNA localization

Applied decision tree learning (Breiman et al., 1984) was applied on various data groups using the R package rpart (Therneau and Atkinson, 2018) with RNA localization as a binary response. The ratio of localized to non-localized dataset is \(1:9\), so Cohen’s Kappa coefficient was used to assess the agreement between the model’s prediction and observed data. Different weights were assigned to localizing and non-localizing mRNAs to ensure the model emphasize on getting localizing mRNAs correct. To obtain an optimal weight, we performed 1000 bootstraps (with replacement) across all data groups and in each bootstrap, we ensure that the non-localizing and localizing ratios are preserved. This bootstrapping was used for subsequent analysis as well. Based on getting the average Kappa across all data groups, a final weight of 1 for non-localizing mRNA and 3.4 for localizing mRNA was used.

QUANTIFICATION AND STATISTICAL ANALYSIS

Software and statistical analysis details can be found in the Key Resources Table, Results, and Computational data processing section (see above). All statistical analyses were performed in R using the stats package (version 3.3.3 and 3.5.0), with the numbers tested indicated in the main or supplementary figures. Changes in EJC enrichment were analyzed using the DESeq2 package (Love et al., 2014). Two-sided t-test for group comparisons were performed using the t.test() function, correlation was estimated using the cor.test() function and the ANOVA analysis used the lm() and anova() functions. Linear regression was performed using lm() function and t-statistics for each coefficient was obtained using the summary.lm() function. Boxplot elements show the median (black line) and quantile values (box denotes 25th and 75th quantile), with outliers shown as black dots outside of the box whiskers. Violin plots show median (black dot), 25th and 75th quantile (black line) and distribution of the groups. For Figures 6A and 6A, 2275 significantly enriched junctions (FDR < 0.05 and delta log₂-fold change > log2(1.5)) were tested against 4334 depleted junctions (FDR < 0.05 and delta log₂-fold change > log2(1.5)). For Figure 6C, the number of junctions in each folding category is as follows: 1. 10220, 2. 2220, 3. 1009, 4.1737.

DATA AND CODE AVAILABILITY

The accession number for the FASQ files of ipaRt, mRBP footprinting and mRNA-Seq reported in this paper is European Nucleotide Archive PRJEB26421: https://www/ebi.ac.uk/ena/data/PRJEB26421.