

A regulator of *Dscam* mutually exclusive splicing fidelitySara Olson¹, Marco Blanchette^{2,3,6}, Jung Park¹, Yiannis Savva^{1,6}, Gene W Yeo⁴, Joanne M Yeakley⁵, Donald C Rio^{2,3} & Brenton R Graveley¹

The *Down syndrome cell adhesion molecule (Dscam)* gene has essential roles in neural wiring and pathogen recognition in *Drosophila melanogaster*. *Dscam* encodes 38,016 distinct isoforms via extensive alternative splicing. The 95 alternative exons in *Dscam* are organized into clusters that are spliced in a mutually exclusive manner. The exon 6 cluster contains 48 variable exons and uses a complex system of competing RNA structures to ensure that only one variable exon is included. Here we show that the heterogeneous nuclear ribonucleoprotein hrp36 acts specifically within, and throughout, the exon 6 cluster to prevent the inclusion of multiple exons. Moreover, hrp36 prevents serine/arginine-rich proteins from promoting the ectopic inclusion of multiple exon 6 variants. Thus, the fidelity of mutually exclusive splicing in the exon 6 cluster is governed by an intricate combination of alternative RNA structures and a globally acting splicing repressor.

Pre-mRNA splicing is frequently used to regulate important biological processes. It is currently estimated that between 60% and 75% of human genes encode alternatively spliced pre-mRNAs^{1,2}. Though most of these genes encode 2–3 different isoforms, there are several examples of genes that can generate much larger repertoires of mRNAs. For example, the human *KCNMA1* gene can generate ~500 different isoforms, and the three *NRXN* genes together produce over 2,000 isoforms^{3,4}. However, by far the most notable example is the *D. melanogaster Dscam* gene, which can generate 38,016 isoforms⁵.

Dscam encodes a diverse collection of transmembrane domain receptors that have an important role in neural wiring and pathogen recognition⁶. For example, in the nervous system, *Dscam* is required for the proper wiring of Bolwigs nerve⁵, olfactory receptor neurons⁷, projection neurons of the olfactory system⁸, mushroom body neurons^{9–11} and mechanosensory neurons¹². In every case that has been examined, mutations in *Dscam* result in neural wiring defects. More recently, *Dscam* has been shown to play an important role in the insect immune system. In both *D. melanogaster*¹³ and the mosquito *Anopheles gambiae*¹⁴, *Dscam* is required to mount an effective immune response.

In *D. melanogaster*, *Dscam* contains 115 exons, of which 95 are alternatively spliced⁵ (Fig. 1a). These variable exons are organized in clusters; the exon 4, 6, 9 and 17 clusters contain 12, 48, 33 and 2 variable exons, respectively. Importantly, the exons within each cluster are spliced in a mutually exclusive manner—only one exon from each cluster is included in the mRNA. The exon 4, 6 and 9 clusters encode alternative versions of extracellular immunoglobulin repeats, whereas

the exon 17 cluster encodes two different transmembrane domains, such that each of the 38,016 isoforms has the same overall domain structure.

From a mechanistic view one of the most puzzling aspects of *Dscam* is how the splicing of the large clusters of exons occurs in a mutually exclusive manner. Thousands of eukaryotic genes contain regions that have two mutually exclusive exons, and a variety of mechanisms are known that ensure that only one exon is included in these mRNAs. These mechanisms involve steric hindrance, the use of both the major and minor spliceosomes, and the nonsense-mediated decay pathway¹⁵. However, none of these mechanisms explain how the variable clusters in *Dscam* can be spliced in a mutually exclusive manner.

Insight into the mechanism by which *Dscam* splicing occurs in a mutually exclusive manner was obtained from comparative genomics^{16,17}. The exon 6 cluster contains two classes of conserved elements: the docking site, which is located in the intron downstream of constitutive exon 5, and the selector sequences, which are located upstream of each of the 48 exon 6 variants. Each selector sequence is complementary to a portion of the docking site, and this pairing juxtaposes one, and only one, alternative exon to the upstream constitutive exon. The mutually exclusive nature of the docking site-selector sequence interactions immediately suggests that the formation of these competing RNA structures is a central component of the mechanism guaranteeing that only one exon 6 variant is included in each *Dscam* mRNA. Notably, it seems as though a different mechanism is used to ensure that the exon 4 and 9 clusters are spliced in a mutually exclusive manner.

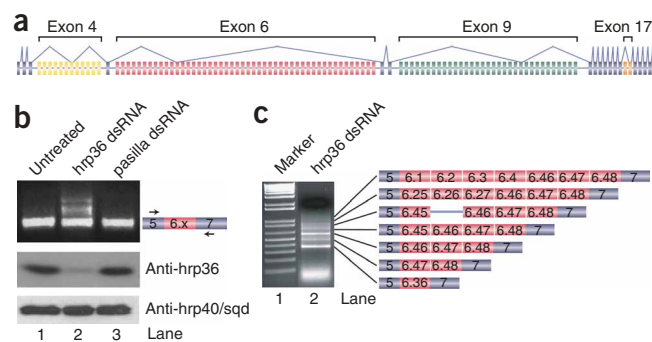
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Figure 1 *hrp36* is required for mutually exclusive splicing fidelity in the exon 6 cluster of *Dscam*. (a) Organization of *D. melanogaster Dscam*. (b) S2 cells were incubated alone or in the presence of *hrp36* or *pasilla* double-stranded RNA, and splicing of the exon 6 cluster was examined by RT-PCR using primers in exons 5 and 7. Below, western blot showing that *hrp36* levels are reduced in cells treated with *hrp36* double-stranded RNA and that depletion of *hrp36* is specific, as the levels of *hrp40/sqd* are unchanged. (c) A higher resolution gel, cloning and sequencing revealed that the larger bands correspond to products derived from mRNAs containing multiple exon 6 variants.



Although the mutually exclusive nature of docking site-selector sequence interactions suggests that they have a key role in ensuring that only one exon 6 variant is included in each mRNA, the mechanism by which these RNA structures do this is unknown. Moreover, these RNA structures alone cannot explain why the variable exons whose selector sequences do not interact with the docking site are not included. This has prompted us to search for proteins that have a role in ensuring the fidelity of exon 6 mutually exclusive splicing. Here, we show that the heterogeneous nuclear ribonucleoprotein (hnRNP) *hrp36* has a key role in this process—depletion of *hrp36* by RNA interference results in the inclusion of multiple exon 6 variants. The effects of *hrp36* are cluster wide (*hrp36* depletion affects most if not all exons within the exon 6 cluster) and cluster specific (*hrp36* depletion has no effect on the mutually exclusive splicing of the exon 4, 9 or 17 clusters). Moreover, we find that *hrp36* functions by preventing serine/arginine-rich (SR) proteins from promoting the ectopic inclusion of multiple exon 6 variants. These results add a new layer to the mechanism that ensures that only one exon 6 variant is included in the *Dscam* mRNA.

RESULTS

hrp36 ensures exon 6 mutually exclusive splicing fidelity

To identify proteins involved in controlling *Dscam* exon 6 mutually exclusive splicing, we performed an RNAi screen in which *D. melanogaster* S2 cells were treated with various double-stranded RNAs and *Dscam* splicing was monitored first by a fiber optic universal microarray^{18,19} containing 1,536 probes (including probes for all of the *Dscam* exons) and second by reverse transcription PCR (RT-PCR) using primers in exons 5 and 7 of *Dscam* followed by agarose gel electrophoresis. For the RNAi screen we used a library of ~400 double-stranded RNAs corresponding to genes encoding RNA-binding proteins, spliceosomal components and DEXH/D-box ATPases²⁰. The microarray screen identified seven proteins as candidates for controlling the fidelity of mutually exclusive splicing, as they modulated the splicing of most if not all exons within at least one of the clusters (**Supplementary Discussion and Supplementary**

Fig. 1 online). These include both subunits of U2AF (*CG9998/dU2AF50* and *CG3582/dU2AF38*), dCAPER/dCC1.3 (*CG11266/CC1.3*), the spliceosomal DEXH/D-box ATPases dBrr2 (*CG5931/Brr2*), dPrp5 (*CG6197/Prp5*) and Rm62 (*CG10279/Rm62/p68*), and the hnRNP *hrp36* (*hrp36/Hrb87F/CG12749*). However, our secondary RT-PCR screen using primers in exons 5 and 7 revealed that only *hrp36* double-stranded RNA has an impact on exon 6 mutually exclusive splicing (**Fig. 1b**). We identified RT-PCR products larger than those containing only a single exon 6 variant when using RNA isolated from cells depleted of *hrp36* (**Fig. 1b**, lane 2), but not when using RNA from untreated cells (**Fig. 1b**, lane 1) or from cells treated with a control double-stranded RNA (*pasilla*, encoded by *ps*) (**Fig. 1b**, lane 3). Cloning and sequencing of these products revealed that they are derived from RNAs containing multiple exon 6 variants spliced together (**Fig. 1c**). We identified products that include up to seven exon 6 variants, though larger products can be observed on gel, which suggests that vast numbers of exon 6 variants can be spliced together in the absence of *hrp36*. In most cases, adjacent exons are spliced together (for example, exon 6.45 is spliced to 6.46), though we also identified clones in which exons located

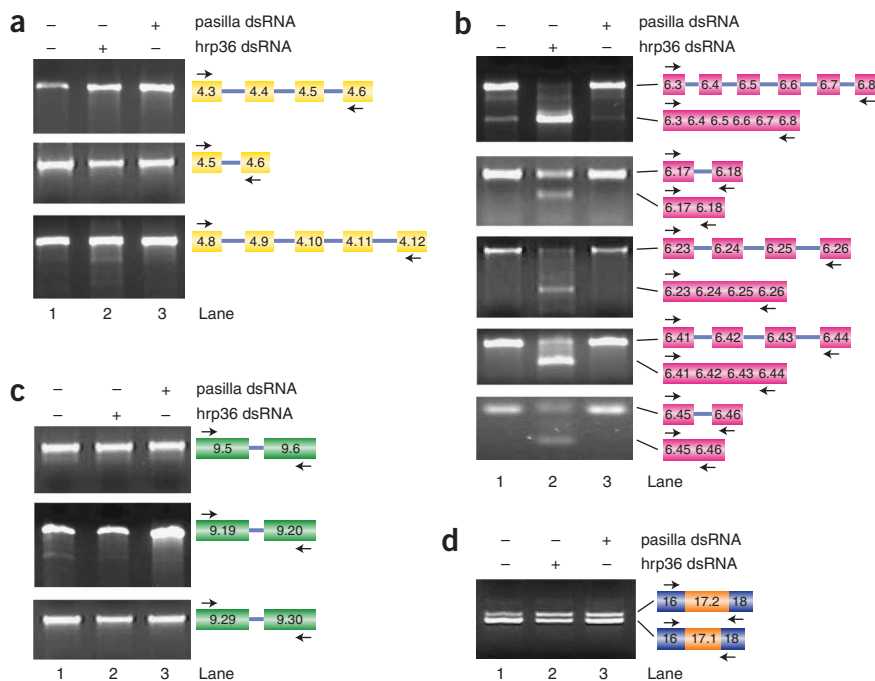


Figure 2 *hrp36* functions in a cluster-specific manner. (a–d) RT-PCR was used to examine the role of *hrp36* in mutually exclusive splicing of the exon 4 (a), exon 6 (b), exon 9 (c) and exon 17 (d) clusters. These experiments revealed no impact of *hrp36* depletion on mutually exclusive splicing of the exon 4, exon 9 or exon 17 clusters. In contrast, depletion of *hrp36* results in the splicing of multiple adjacent exon 6 variants throughout the exon 6 cluster. The primers used in each experiment and the identity of the products, which were confirmed by cloning and sequencing, are depicted.

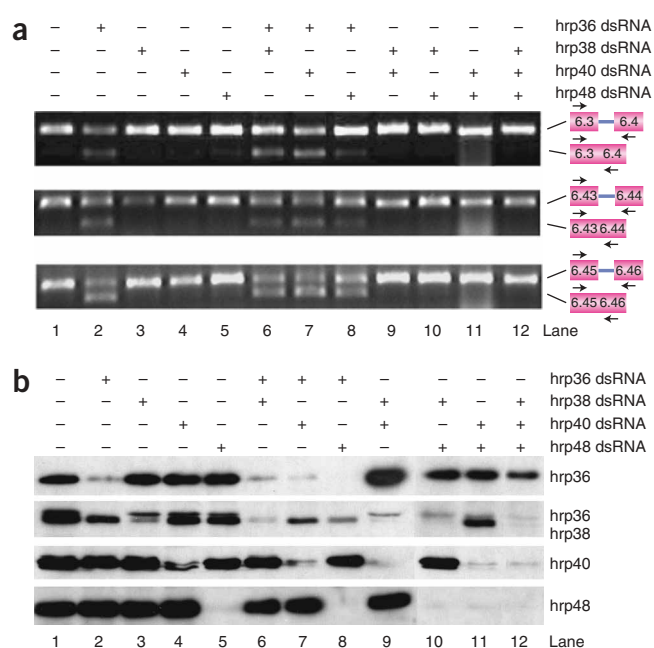


Figure 3 hrp36 is the only major hnRNP protein that regulates the fidelity of exon 6 mutually exclusive splicing. **(a,b)** *D. melanogaster* S2 cells were treated with hrp36, hrp38, hrp40 and hrp48 double-stranded RNAs as indicated. Exon 6 splicing was examined by RT-PCR with primers to detect the specified exons **(a)**, and the extent and specificity of depletion was monitored by western blotting **(b)**. Note that the hrp38 antibody cross-reacts with hrp36 to some extent.

far from one another in the pre-mRNA were spliced together (for example, exon 6.4 is spliced to exon 6.46). The observation that adjacent exons were frequently spliced together in the absence of hrp36 indicates that there are no inherent *cis*-acting physical barriers, such as intron size, that normally prevent adjacent exon 6 variants from being spliced to one another. Importantly, the correct exon 6 splice sites were used in all but one of the clones sequenced. In this case, the clone also contained the intron-separating exons 6.45 and 6.46. Consistent with the microarray results (**Supplementary Fig. 1**), most of the clones contained exons 6.46, 6.47 and 6.48, either alone or in combination with other exons. The effect of depleting hrp36 on

exon 6 mutually exclusive splicing is not due to an off-target RNAi effect, as identical results are obtained using a second, nonoverlapping double-stranded RNA corresponding to the 3' untranslated region of the hrp36 mRNA (**Supplementary Fig. 2** online). Together, these data indicate that hrp36 has a key role in ensuring the fidelity of exon 6 mutually exclusive splicing in S2 cells.

hrp36 acts in a cluster-specific and cluster-wide manner

We next tested whether hrp36 has a role in ensuring the fidelity of mutually exclusive splicing in the other alternatively spliced clusters in *Dscam* (**Fig. 2**). Previously, we have shown that hrp36 has a role in alternative splicing of the exon 4 and 17 clusters of *Dscam*, but in these cases hrp36 controls which of the variable exons are selected rather than the number of exons included²⁰. Consistent with these earlier results, when we used constitutive exon primers, we found no evidence that hrp36 RNAi results in the inclusion of multiple variable exons in the exon 4, 9 or 17 clusters (**Fig. 2d**; data not shown).

Although these data suggest that hrp36 does not impact the mutually exclusive splicing of the other clusters, we developed a more sensitive assay that uses primers within the alternatively spliced exons to more rigorously test this. For example, when primers in exons 6.3 and 6.8 are used in RT-PCR reactions with total RNA from untreated cells, or from cells treated with a control double-stranded RNA, we primarily observe products corresponding to unspliced pre-mRNA (presumably amplified from released lariat introns) (**Fig. 2b**, lanes 1 and 3). In contrast, when cells are treated with hrp36 double-stranded RNA, we observe a switch to a smaller product that contains exons 6.3 through 6.8 accurately spliced together (**Fig. 2b**, lane 2). Using this assay we find that hrp36 controls the fidelity of mutually exclusive splicing throughout the entire exon 6 cluster (**Fig. 2b**; data not shown). In contrast, cells depleted of hrp36 do not display defects in mutually exclusive splicing in the exon 4, 9 or 17 clusters (**Fig. 2a,c,d**). These results demonstrate that hrp36 acts in both a cluster-wide and cluster-specific manner to ensure the fidelity of mutually exclusive splicing.

We also tested whether hrp36 is the only hnRNP protein that regulates the fidelity of exon 6 mutually exclusive splicing in S2 cells. To do this we treated S2 cells with double-stranded RNAs to hrp36, hrp38, hrp40 and hrp48 either alone or in combination and examined exon 6 splicing by RT-PCR (**Fig. 3a**) and the extent of depletion of each protein by western blot (**Fig. 3b**). We found that depletion of only hrp36 (**Fig. 3a,b**, lane 2), but not the three other hrp proteins

Table 1 Distribution of hrp36 binding sites in the *Dscam* exon 6 cluster in 12 *Drosophila* species

Species	Number of exon 6 variants	Number of exonic hrp36 sites	Number (%) of exons with ≥ 1 hrp36 site	Size of all exons (base pairs)	Exonic sites/kilobase	Number of intronic hrp36 sites	Number (%) of introns with ≥ 1 hrp36 site	Size of all introns (base pairs)	Intronic sites/kilobase	Fold enrichment exon/intron
<i>D. melanogaster</i>	48	46	29 (60.4%)	5,901	7.80	22	14 (28.6%)	6,842	3.22	2.42
<i>D. simulans</i>	48	40	25 (52.1%)	5,901	6.78	20	10 (20.4%)	6,757	2.96	2.29
<i>D. yakuba</i>	41	43	25 (61.0%)	5,039	8.53	15	9 (21.4%)	6,692	2.24	3.81
<i>D. sechellia</i>	47	45	29 (61.7%)	5,785	7.78	20	10 (20.8%)	6,842	2.92	2.66
<i>D. erecta</i>	45	47	31 (68.9%)	5,541	8.48	19	13 (26.3%)	6,566	2.89	2.93
<i>D. ananassae</i>	46	55	28 (60.9%)	5,666	9.71	14	6 (12.8%)	6,168	2.27	4.28
<i>D. pseudoobscura</i>	48	69	34 (60.8%)	5,913	11.67	30	11 (22.4%)	6,881	4.36	2.68
<i>D. persimilis</i>	48	68	35 (72.9%)	5,909	11.51	30	12 (24.5%)	6,973	4.30	2.67
<i>D. willistoni</i>	49	38	29 (59.2%)	6,028	6.30	7	3 (6.0%)	5,927	1.18	5.34
<i>D. mojavensis</i>	50	60	40 (80.0%)	6,156	9.75	17	12 (23.5%)	7,685	2.21	4.41
<i>D. virilis</i>	52	53	36 (69.2%)	6,548	8.09	20	10 (18.9%)	7,692	2.60	3.11
<i>D. grimshawi</i>	53	49	32 (60.4%)	6,522	7.51	18	14 (25.9%)	8,040	2.24	3.36

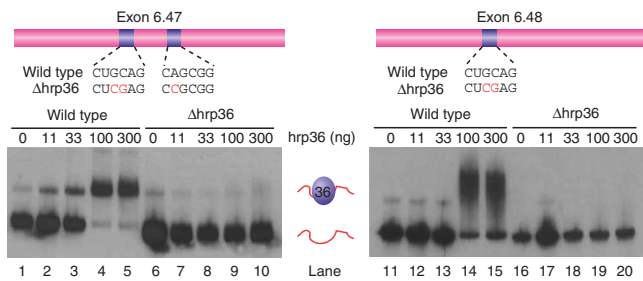


Figure 4 hrp36 binds to exon 6 variants. Gel mobility shift experiments show that exons 6.47 (left) and 6.48 (right) bind to recombinant hrp36. However, mutations that disrupt the putative hrp36 binding sites eliminate the protein-RNA interaction.

(Fig. 3a,b, lanes 3–5), results in the inclusion of multiple exons. Moreover, co-depletion of hrp36 and either hrp38, hrp40 or hrp48 did not enhance the effect of depleting hrp36 alone (Fig. 3a,b, lanes 6–8). Additionally, the simultaneous depletion of hrp38, hrp40 and hrp48 had no impact on exon 6 splicing (Fig. 3a,b, lane 12). Thus, hrp36 is the only major *D. melanogaster* hnRNP protein that has a role in the fidelity of exon 6 mutually exclusive splicing in S2 cells.

hrp36 binds throughout the exon 6 cluster

To begin to examine possible mechanisms by which hrp36 could function to ensure the fidelity of exon 6 alternative splicing of the entire exon 6 cluster, we have examined the interaction between hrp36 and the *Dscam* pre-mRNA. First, we used a position-specific scoring matrix (PSSM) derived from systematic evolution of ligands by exponential enrichment (SELEX) experiments with hrp36 (M.B., R. Green, S. MacArthur, M. Eisen and D.C.R., unpublished data) to scan the entire *D. melanogaster Dscam* pre-mRNA for potential hrp36 binding sites. We identified numerous potential hrp36 binding sites throughout the *Dscam* gene. Within the exon 6 cluster we found that the hrp36 binding sites are significantly enriched in exons and depleted from introns ($P = 0.0007$) (Table 1). For example, 29 of the 48 exon 6 variants in *D. melanogaster* contain at least one hrp36 binding site, whereas only 14 of the 49 introns flanking the exon 6 variants contain a hrp36 site. Importantly, this trend is conserved in all 12 *Drosophila* species that have been sequenced ($P = 0.0003$). The most extreme case is the *D. mojavensis Dscam* gene, in which 40 of the 50 exon 6 variants contain at least one hrp36 site, whereas only 12 of the 51 introns do (Table 1). These results suggest that hrp36 may promote the fidelity of mutually exclusive splicing by binding to the exon 6 variants.

To examine the potential interaction between hrp36 and the exon 6 variants, we performed gel mobility shift assays. For these experiments we tested exon 6.47, which contains two potential hrp36 binding sites, and exon 6.48, which contains one potential hrp36 binding site. ^{32}P -labeled RNAs containing the entire exon sequence (123 nucleotides) were synthesized and incubated with recombinant hrp36, and the reactions were resolved on nondenaturing polyacrylamide gels. We found that hrp36 binds to both exon 6.47 and 6.48 (Fig. 4, lanes 1–5 and 11–15). However, point mutations that

disrupt the integrity of the hrp36 binding sites prevent these interactions (lanes 6–10 and 16–20). Thus, hrp36 can physically interact with the exon 6 variants in a sequence-specific manner *in vitro*.

To analyze whether hrp36 interacts with the *Dscam* pre-mRNA *in vivo*, we performed RNP immunoprecipitation-Chip (RIP-Chip) assays (M.B., R. Green, S. MacArthur, M. Eisen and D.C.R., unpublished data). Briefly, antibodies to hrp36 (and hrp38, hrp40 and hrp48 as controls) were used to immunoprecipitate RNP complexes from *D. melanogaster* S2 cell nuclear extracts. The RNAs isolated from these RNP complexes were then labeled and hybridized to 35-base-pair-resolution tiling arrays covering the entire *D. melanogaster* genome. The hybridization intensities of the RNP-associated RNAs were compared to the starting undepleted nuclear extract, and immunoprecipitations were performed using a nonspecific antibody. Although each hnRNP protein binds to the *Dscam* pre-mRNA to some extent, the pattern is distinct for each protein (Fig. 5). Most notably, hrp36 associated with the entire exon 4, 6 and 17 clusters, but not the exon 9 cluster. This is consistent with the idea that hrp36 has a role in regulating exon choice in the exon 4 and 17 clusters²⁰ and mutually exclusive splicing of the exon 6 cluster while having no impact on the splicing of the exon 9 cluster. Thus, these results, in particular the association of hrp36 throughout the entire exon 6 cluster, are consistent with the observed effects of hrp36 on *Dscam* splicing. Moreover, the functional, bioinformatic, genomic and biochemical data together strongly suggest that hrp36 binds to multiple sites throughout the entire exon 6 cluster to repress the inclusion of multiple exon 6 variants, thus ensuring that only one exon is spliced into the mRNA.

hrp36 prevents SR proteins from activating exon inclusion

Human hnRNP A1, which is closely related to hrp36, has been shown to repress splicing in some cases by binding throughout an exon²¹, where it prevents SR proteins from binding to the exons and promoting their inclusion. Based on this observation, we hypothesized that hrp36 may repress the inclusion of multiple exon 6 variants by preventing the ability of SR proteins to bind to the exons and promote their inclusion. A prediction of this model is that co-depleting an SR protein and hrp36 might reverse the effect of depleting hrp36 alone.

We tested this hypothesis by treating S2 cells with hrp36 double-stranded RNA either alone or in combination with different SR or SR-related protein double-stranded RNAs. Co-depletion of the SR protein B52 (Fig. 6a, lane 4) completely, or nearly completely, reversed the effect of depleting hrp36 (lane 2). Similar but weaker reversal was observed in cells treated with SC35 double-stranded RNA (lane 6), and to a lesser extent with SF2 (lane 7), RBP1-like (lane 3), SRm300 (lane 9) and SRm160 double-stranded RNAs (lane 10). No effect was

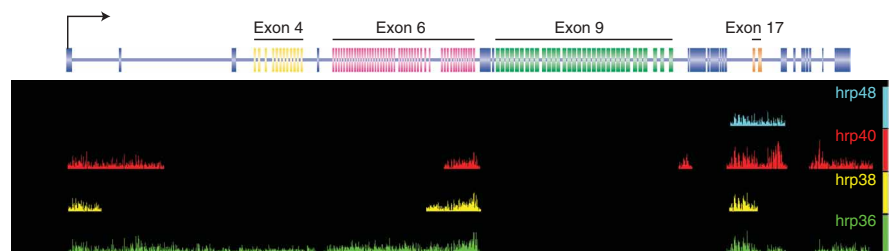


Figure 5 hrp36 binds to *Dscam* pre-mRNA throughout the entire exon 6 cluster. The distribution of the RNA sequences bound by hrp36, hrp38, hrp40 and hrp48 across the *Dscam* gene are shown. These maps were determined by labeling and hybridizing RNAs that coimmunoprecipitate with antibodies specific to each hnRNP protein to genome-wide *Drosophila* tiling arrays.

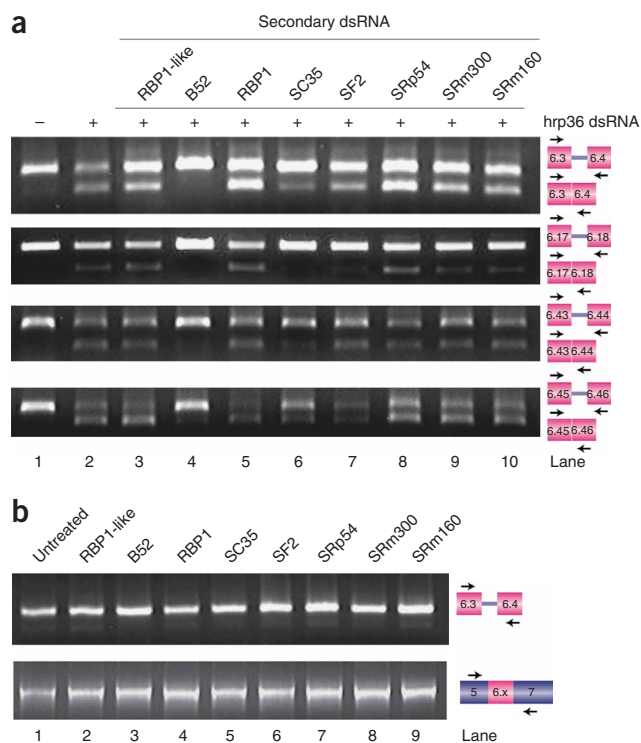


Figure 6 hrp36 prevents ectopic exon 6 inclusion by SR proteins. To test whether the effect of depleting hrp36 could be reversed by co-depleting various SR proteins, S2 cells were treated with double-stranded RNAs to hrp36 and/or the indicated SR proteins. **(a)** The inclusion of multiple exon 6 variants induced by depletion of hrp36 can be reversed to various extents by co-depleting different SR proteins. **(b)** Depletion of the SR proteins alone has no effect on exon 6 mutually exclusive splicing in the presence of hrp36.

observed in cells treated with RBP1 (lane 5) or SRp54 double-stranded RNAs (lane 8). These results are consistent with the hypothesis that in the absence of hrp36, SR proteins promote the inclusion of multiple exon 6 variants.

An alternative explanation for these observations is that the depletion of SR proteins compromises the general efficiency of splicing and consequently decreases the probability that multiple exon 6 variants would be included. If this were the case, we should observe exon 6 skipping in cells that contain hrp36 but are depleted of SR proteins by RNAi. To test this, we examined exon 6 splicing in cells treated with the SR protein double-stranded RNAs, but not with hrp36 double-stranded RNA, by RT-PCR with primers in exons 6.3 and 6.4 (**Fig. 6a**, top) or exons 5 and 7 (**Fig. 6b**, bottom). Importantly, we did not observe any defects in the exon 6 mutually exclusive splicing mechanism in cells treated with any of the SR protein double-stranded RNAs alone. This suggests that treating cells with the SR protein double-stranded RNAs does not result in a general decrease in splicing efficiency. Together, our results suggest that hrp36 ensures the fidelity of exon 6 mutually exclusive splicing by preventing the ability of SR proteins to promote exon 6 inclusion.

DISCUSSION

We have shown that the hnRNP protein hrp36 serves to prevent the inclusion of multiple exon 6 variants. There are several noteworthy features about the manner by which hrp36 functions to ensure the

fidelity of mutually exclusive splicing. First, hrp36 acts throughout the entire exon 6 cluster and simultaneously controls the splicing of a large collection of exons. Second, the role of hrp36 in mutually exclusive splicing is restricted to the exon 6 cluster; hrp36 has no effect on the mutually exclusive splicing of the exon 4, 9 or 17 clusters. Finally, hrp36 binds to most if not all of the exons within the exon 6 cluster. The fact that the exon 6 variants are accurately spliced together in the absence of hrp36 indicates that there are no *cis*-acting physical barriers that prevent this from occurring under normal circumstances. Thus, our data suggest that the binding of hrp36 to the exon 6 variants represses the exon 6 variants from being spliced together.

We have further shown that hrp36 prevents SR proteins from promoting ectopic exon 6 inclusion; depleting different SR proteins, B52 in particular, could in fact restore normal mutually exclusive splicing of the exon 6 cluster to cells that were also depleted of hrp36. Given the well-established antagonism between hnRNP proteins and SR proteins^{21,22}, the simplest explanation for these data is that when hrp36 is bound to the exon 6 variants, it prevents SR proteins from activating their splicing, perhaps by preventing the SR proteins from binding to the exons. Alternatively, as SR proteins are known to have exon-independent functions²³, it is also possible that B52 functions without binding to the exons.

To examine the possibility that B52 promotes exon 6 inclusion by binding to the exon 6 variants, we searched the exon 6 cluster for matches to the B52 consensus sequence determined by SELEX (ref. 24). No matches to this sequence were found in the entire *Dscam* pre-mRNA. However, the properties of the B52 SELEX consensus are different from the SELEX consensus sequences determined for other SR proteins²³. Thus, the B52 SELEX consensus is not likely to represent the full repertoire of potential B52 binding sites. Further work will therefore be needed to determine whether B52 binds to the exon 6 variants in the absence of hrp36.

Depleting hrp36 in S2 cells has a profound effect on exon 6 splicing, and little if any functional *Dscam* protein should be produced under such circumstances. As *Dscam* is essential for viability⁵, it is surprising that flies lacking hrp36 are viable^{25,26} and that exon 6 splicing occurs normally in *hrp36*-null flies (S.O. and B.R.G., unpublished data). This apparent discrepancy is not due to an RNAi off-target effect, as two nonoverlapping hrp36 double-stranded RNAs have the same effect on both exon 6 mutually exclusive splicing and hrp36 levels in S2 cells (**Supplementary Fig. 1**). Thus, there is a true difference in the requirement for hrp36 in exon 6 mutually exclusive splicing between S2 cells and flies. There are many possible reasons that *Dscam* splicing occurs normally in *hrp36* mutants. One possibility is that *D. melanogaster* individuals express one or more proteins that are functionally redundant with hrp36 that are not expressed in S2 cells. Alternatively, as the effect of depleting hrp36 can be reversed by co-depleting SR proteins, such as B52, it could be that the balance between these factors is either different in flies and S2 cells, or is compensated for in the *hrp36* mutant flies. Additionally, the requirement of hrp36 for *Dscam* splicing may be tissue-specific; some cells or tissues may require hrp36, whereas hrp36 may be dispensable in other cells and tissues. Nonetheless, our results clearly demonstrate that hrp36 is essential for *Dscam* splicing in S2 cells. Further experiments will be required to analyze the role of hrp36 in *Dscam* splicing in *D. melanogaster* in more detail and to identify other proteins that functionally substitute for this function of hrp36.

The results reported here support and extend our previously proposed model for the mechanism of exon 6 mutually exclusive splicing¹⁷ (**Fig. 7**). We propose that hrp36 binds to most if not all of

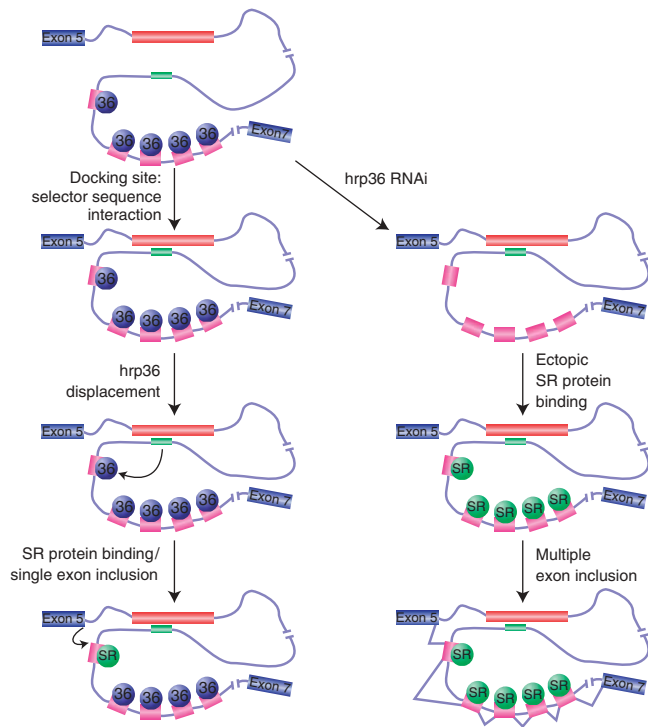


Figure 7 Model for the mechanism by which hrp36 prevents the inclusion of multiple exon 6 variants. hrp36 (blue circles) binds to all of the exon 6 variants (magenta) and represses their inclusion. When the selector sequence upstream of a specific exon interacts with the docking site of hrp36 on the exon immediately downstream, this results in the de-repression of hrp36 on the exon immediately downstream, but not for the other exon 6 variants. In this way, only a single exon 6 variant is included. hrp36 competes with SR proteins (green circles) for binding to the exon 6 variants. In the absence of hrp36, these activators could bind to all of the exon 6 variants and enhance their splicing to one another.

tissue- or cell-specific manner by the expression of specific positive or negative regulators that could either enhance or repress the inclusion of specific exons.

METHODS

RNAi microarray screen. Transcription templates were generated by PCR from a plasmid library containing complementary DNA fragments from ~400 genes encoding mRNA processing factors²⁰. Double-stranded RNA was synthesized by *in vitro* transcription (Epicentre T7 flash kit) and annealed by incubating at 100 °C for 2 min and slowly cooling the reactions to room temperature (20–22 °C) over a 40-min time period. 40 µg of double-stranded RNA was added to each well of a 6-well culture dish containing 2×10^6 *D. melanogaster* S2 cells in *D. melanogaster* SFM medium (Invitrogen) supplemented with l-glutamine and Pen/strep. The cells were grown at 27 °C for 2 d, after which an additional 40 µg of double-stranded RNA was added to each well, and the incubation continued for 2 more d. Total RNA was isolated from each sample with Trizol (Invitrogen) according to the manufacturer's instructions.

Microarray experiments were performed using the DASL assay¹⁸ with a pool of oligonucleotides complementary to 1,536 constitutive or alternative exons in ~200 *D. melanogaster* pre-mRNAs. Between 2 and 10 biological replicates per double-stranded RNA were performed. After normalization of the data and averaging of replicate samples with a correlation coefficient greater than 0.9, Z-scores were calculated for each exon in each sample. Details of these experiments and a global analysis of the results will be described elsewhere.

RT-PCR. Total RNA was isolated from S2 cells using Trizol reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription was performed on each RNA sample using 200 units of Superscript II (Invitrogen) and 5 µg of RNA in a 20 µl reaction, at 42 °C for 1 h. PCR reactions were then set up to analyze alternative splicing of various regions of *Dscam*. Primer sequences are available upon request.

Western blots. Protein was harvested from S2 cells in $1 \times$ SDS-PAGE buffer, heated to 100 °C and then resolved on a 12.5% (w/v) SDS-PAGE gel. Proteins were then electoblotted to a PVDF membrane (Biorad) and blocked in 5% (w/v) nonfat dry milk in Tris-buffered saline/Tween (TBST) at room temperature for 1 h. The primary antibody was added directly to the blocking reagent and incubated overnight at room temperature. Blots were washed in TBST and incubated with the secondary antibody linked to horseradish peroxidase for 1 h at room temperature. Western blots were developed using Western Lightning chemiluminescence kit (Perkin Elmer).

Gel-shift assays. RNAs corresponding to exons 6.47 and 6.48, or variants of these exons in which point mutations were generated to disrupt the putative hrp36 binding sites, were synthesized by *in vitro* transcription with α -³²P-UTP and gel purified. The RNAs were incubated with the indicated quantities of hrp36 that were expressed *in vitro* and purified from *Escherichia coli*. After incubation for 15 min at room temperature, heparin was added to a final concentration of 0.5 mg ml⁻¹, and the reactions were resolved on a 5% (w/v) (29:1) acrylamide gel. The gel was then dried and visualized by autoradiography.

Identification of hrp36 binding sites in *Dscam*. A PSSM for the hrp36 binding site derived from SELEX experiments was used to search the *Dscam* gene from 12 *Drosophila* species¹⁷. We used a raw score of 5.0 as a cutoff for putative hrp36 binding sites. The location of each site within the exon 6 cluster was used to determine the frequency of sites within exons and introns for each

the exon 6 variants and represses their inclusion. When a selector sequence interacts with the docking site, something about that interaction causes the hrp36 molecules bound to the downstream exon to either dissociate from the exon or become unable to repress splicing of that exon. This would then allow for SR proteins to bind the exon and promote its inclusion. As the docking site-selector sequence interaction would only act on the selected exon, all of the downstream exons would remain repressed by hrp36 and could not be included. This model explains why multiple exons are included when hrp36 is depleted: in the absence of hrp36, all of the exon 6 variants can be included because none of the exons are repressed.

While this model explains many aspects of how splicing of the exon 6 cluster occurs in a mutually exclusive manner, there remain many questions. First of all, how does the docking site-selector sequence interaction relieve the repression exerted by hrp36 on the downstream exon? What determines which of the 48 selector sequences interacts with the docking site in a given pre-mRNA? Is this process highly regulated or does it occur in a random or stochastic manner? Microarray experiments have revealed little if any regulation of exon 6 splicing^{11,27}, which is consistent with the biology of *Dscam*. In the nervous system, it seems that the actual identity of the *Dscam* isoforms that are expressed is unimportant, provided that each neuron expresses a different set of isoforms than the neighboring neurons^{11,28–31}. Similarly, within the immune system, where *Dscam* functions in a manner analogous to vertebrate antibodies^{13,16}, hemocytes must be capable of expressing most if not all of the potential *Dscam* isoforms to mount an effective immune defense against invading pathogens. The simplest way to accomplish these tasks in both the nervous and immune systems would be via a stochastic mechanism. It is therefore noteworthy that each exon 6 variant is repressed by hrp36 and contains a selector sequence that can interact with the docking site. Thus, the exon 6 cluster is organized such that even if exon selection occurs randomly, splicing would still occur in a mutually exclusive manner. In addition, the stochastic splicing of the exon 6 cluster could easily be overridden in a developmental-

species. The statistical significance of the findings was evaluated by performing a chi-squared test on the differential enrichment of the hrp36 sites in exons versus introns.

RIP-Chip assays. Nuclear RNP fractions from S2 cells were prepared using a modified version of the mammalian RNP protocol³². Briefly, 16 l of exponentially growing S2 cells were harvested, and their nuclei were isolated by hypotonic lysis following the Dignam extract protocol³³. The nuclei were resuspended in 2.5 packed-cell volumes with ice-cold (2–4 °C) HNEB2 (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 2.5 mM MgCl₂, 0.5% (v/v) Triton X-100, 0.2 mM PMSF) and sonicated 3 times for 30 s. An RNP-enriched fraction was isolated by centrifugation of the sonicated nuclei over a 30% (w/v) sucrose cushion (HNEB2 plus 30% (w/v) sucrose) for 15 min at 4,800g. Immunopurification of the hnRNP-containing RNP complexes was done by mixing 1 ml of the S2 RNP fraction with ~200 ng of affinity-purified rabbit polyclonal antibodies in a total volume of 10 ml of ice-cold IPB1 (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM MgCl₂, 0.05% (v/v) Triton X-100, 0.5 mM PMSF, 1× protease inhibitor cocktail). The antibody/RNP fraction was rocked for 15 min at 4 °C, and the solution was filtered through a 0.2-mm Millex GV filter (Millipore) followed by a 15-min incubation at 4 °C with 0.25 ml of a 50% (v/v) slurry of protein A agarose CL-4B (Sigma). The beads were recovered and washed 4 times with 10 ml of cold (2–4 °C) IPB2 (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM MgCl₂, 0.25% (v/v) Triton X-100) buffer. The RNA was eluted with 350 µl of buffer RLT1 (Qiagen) with β-mercaptoethanol (Qiagen) and purified following the RNeasy midi kit protocol (Qiagen). Total RNA purified from a single immunopurification was amplified and labeled following the GeneChip WT amplified double-stranded cDNA synthesis kit (Affymetrix) and hybridized on a single GeneChip *Drosophila* 1.0R whole-genome tiling array following the manufacturer's recommendations (Affymetrix). These arrays were compared to arrays hybridized with sample isolated from total RNA purified directly from the starting RNP fraction. Downstream data analysis was performed using the TiMAT application suite (<http://bdtncp.lbl.gov/TiMAT/>), and the data were visualized using the Affymetrix Integrated Genome Browser (http://www.affymetrix.com/support/developer/tools/download_igb.affx).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

S.O., M.B., J.P., Y.S. and J.M.Y. performed the experiments; S.O., M.B., J.P., Y.S., J.M.Y., D.C.R. and B.R.G. designed the experiments; S.O., M.B., J.P., Y.S., J.M.Y., G.W.Y., D.C.R. and B.R.G. analyzed the data; B.R.G. wrote the paper with input from all authors.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/nsmb/>.

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