ARTICLES

An RNA map predicting Nova-dependent splicing regulation

Jernej Ule[1,2]*†, Giovanni Stefani[1,2]*†, Aldo Mele[1,2], Matteo Ruggiu[1,2], Xuning Wang[3], Bahar Taneri[4]†, Terry Gaasterland[4]†, Benjamin J. Blencowe[5] & Robert B. Darnell[1,2]

Nova proteins are neuron-specific alternative splicing factors. We have combined bioinformatics, biochemistry and genetics to derive an RNA map describing the rules by which Nova proteins regulate alternative splicing. This map revealed that the position of Nova binding sites (YCAY clusters) in a pre-messenger RNA determines the outcome of splicing. The map correctly predicted Nova's effect to inhibit or enhance exon inclusion, which led us to examine the relationship between the map and Nova's mechanism of action. Nova binding to an exonic YCAY cluster changed the protein complexes assembled on pre-mRNA, blocking U1 snRNP (small nuclear ribonucleoprotein) binding and exon inclusion, whereas Nova binding to an intronic YCAY cluster enhanced spliceosome assembly and exon inclusion. Assays of splicing intermediates of Nova-regulated transcripts in mouse brain revealed that Nova preferentially regulates removal of introns harbouring (or closest to) YCAY clusters. These results define a genome-wide map relating the position of a *cis*-acting element to its regulation by an RNA binding protein, namely that Nova binding to YCAY clusters results in a local and asymmetric action to regulate spliceosome assembly and alternative splicing in neurons.

Genome-wide identification of DNA and RNA regulatory elements has provided insights into the combinatorial nature of regulatory networks[1–7]. However, genome-wide rules relating the position of regulatory elements to their differential activity have not been defined. For example, bioinformatic studies have identified several *cis*-acting RNA motifs that regulate splicing[3,5–7], but the *in vivo* relationship between the position of these motifs in pre-mRNA and the activity of RNA-binding proteins that recognize them is limited[4,8–10]. This issue is of great interest given the role of splicing regulation in tissue-specific functions, particularly in neurons[11,12].

The first tissue-specific splicing factors described in vertebrates were the neuronal RNA-binding proteins Nova1 and Nova2 (refs 13–15). Clusters of an RNA motif, YCAY (where Y indicates a pyrimidine), defined by *in vitro* RNA selection and X-ray crystallography[16–18], were necessary and sufficient to confer Nova-dependent regulation on three alternatively spliced target transcripts[14,19,20]. Recently we developed two technologies, cross-linking and immunoprecipitation (CLIP)[21] and a splicing microarray[22], that identified over 50 Nova-regulated exons; these were validated in *Nova1*$^{-/-}$ or *Nova2*$^{-/-}$ mouse brain. Here we find that the distribution of YCAY clusters in Nova target pre-mRNAs delineates an RNA map that predicts Nova's action on a genome-wide level, and we relate this RNA map to Nova's effect on splicing.

An RNA map of Nova splicing elements

Sequences of pre-mRNAs containing 48 Nova-regulated exons[14,20–22] (Supplementary Tables 1 and 2) were enriched in YCAY motifs near Nova-regulated splice junctions (Fig. 1 and Supplementary Fig. 2). Most YCAY motifs were closely spaced in clusters averaging 28 nucleotides (90%; Supplementary Fig. 3a, b) and were well conserved between mouse and human (97%; Supplementary Fig. 3c). Two-thirds (31 out of 48) of Nova-regulated pre-mRNAs contained conserved YCAY clusters near one of the splice sites, compared with 0.9% of control pre-mRNAs (Supplementary Fig. 4f and Supplementary Tables 1 and 2).

The position of YCAY clusters correlated with Nova's action in splicing regulation (Supplementary Figs 4 and 5). Among the five major locations of YCAY clusters, two predict splicing enhancers (correlating with Nova-dependent exon inclusion; termed Nova intronic splicing enhancers NISE2 and NISE3) and three predict splicing silencers (correlating with Nova-dependent exon skipping; termed Nova intronic splicing silencers NISS1 and NISS2 and Nova exonic splicing silencers (subdivided into NESS1 and NESS2); Fig. 1). The two main Nova splicing enhancers were intronic, located downstream of the alternative exon (NISE2: $n = 18$, 118-fold higher abundance than at the corresponding position in control pre-mRNAs, $P < 0.001$ by two-tailed *t*-test, unequal variance; NISE3: $n = 12$, 119-fold higher abundance, $P = 0.002$). These were further classified as having one (12 out of 21, Supplementary Figs 5b, c and 7 and Supplementary Table 1) or both (9 out of 21, Fig. 1c and Supplementary Table 1) NISE2 and NISE3 elements. In addition, three pre-mRNAs had enhancer YCAY clusters upstream of the alternative exon, in a position analogous to the previously defined NISE1 enhancer element in GlyRα2 pre-mRNA[16] (Fig. 1b; see also Supplementary Fig. 7 and Supplementary Table 2). Nova splicing silencers were either immediately upstream of (NISS2; $n = 6$, 25-fold higher abundance, $P < 0.05$) or within (NESS1, NESS2; $n = 8$, 37-fold higher abundance, $P = 0.01$) the alternative exon, or near the upstream constitutive exon (NISS1; $n = 5$, 70-fold higher abundance, $P = 0.03$; Supplementary Fig. 6a). As a whole, we found five major and two minor positions of YCAY clusters, three of which

[1]Howard Hughes Medical Institute, [2]Laboratory of Molecular Neuro-Oncology, [3]Biocomputing, Information Technology, and [4]Laboratory of Computational Genomics, The Rockefeller University, 1230 York Ave, New York, New York 10021, USA. [5]Banting and Best Department of Medical Research, University of Toronto, Ontario M5G 1L6, Canada. †Present addresses: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK (J.U.); Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut 06511, USA (G.S.); UCSD, Scripps Institution of Oceanography, Scripps Genome Center, 8750 Biological Grade, La Jolla, California 92037, USA (B.T., T.G.); Eastern Mediterranean University, Famagusta, Cyprus (B.T.).
*These authors contributed equally to this work.

mirrored positions seen in previously analysed Nova-regulated transcripts[16,19,20]. We refer to the whole set of positions where YCAY clusters act as Nova-dependent splicing elements as an RNA map (Fig. 1a).

Prediction of Nova-dependent splicing regulation

To test whether the RNA map can predict Nova-dependent splicing regulation *de novo*, we calculated a net YCAY cluster score by subtracting Nova silencer from enhancer cluster scores (Supplementary Fig. 4). Using a stringent scoring method (|net YCAY cluster score| > 2.7), we identified 51 candidate Nova-regulated alternative exons in a genomic database of bioinformatically predicted alternative exons (B.T. and T.G., personal communication). Ten previously validated Nova-regulated exons were among these top predictions. Of the remaining 41 predicted exons, an additional 20 showed significant ($P < 0.01$) splicing change in mouse brain from *Nova1*$^{-/-}$ *Nova2*$^{-/-}$ relative to wild-type littermates (Fig. 2; see also Supplementary Fig. S6, Supplementary Table 3 and http://splicing.rockefeller.edu/map/clusters).

The RNA map correctly predicted the direction of splicing change for 30 out of 30 Nova-regulated exons (14 exons with a score below −2.7 were downregulated, and 16 exons with a score above 2.7 were upregulated; Fig. 2a), including regulated alternative splice sites and alternative terminal exons (Fig. 2b, c; see also Supplementary Figs 6d, e, 7b). We also assessed some predicted YCAY clusters with absolute scores lower than 2.7; although the number of false-positive splicing predictions increased, three additional clusters predicted true Nova-regulated exons, again with correct prediction of splicing change (Supplementary Table 3). Thus, the RNA map accurately predicts splicing silencer and enhancer elements regulated by Nova in mouse brain.

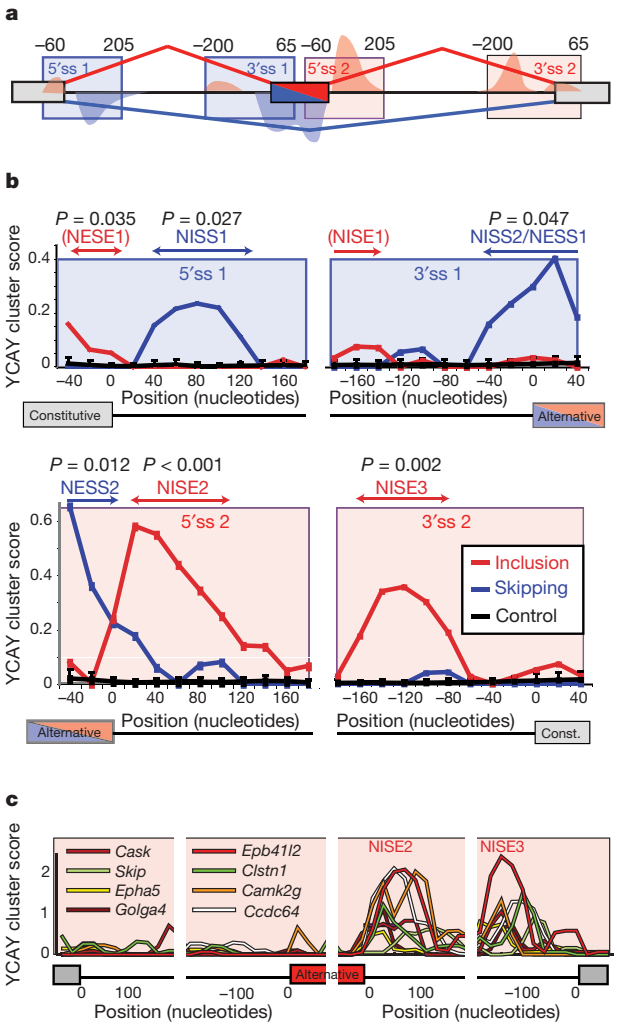

Figure 1 | Definition of the Nova–RNA binding map. **a**, A generic pre-mRNA showing the four regions that define the Nova–RNA binding map (the start and end of each region is labelled by a nucleotide distance to the splice site). Peaks demonstrate the positions of Nova-dependent splicing enhancers (red) or silencers (blue). **b**, A conserved YCAY cluster score (*y* axis) was calculated as described in Supplementary Methods. The *x* axis shows the nucleotide position of the centre of the sequence window. At each significant peak of YCAY cluster enrichment in Nova-regulated versus control pre-mRNAs, the *P*-value (two-tailed *t*-test, unequal variance) of YCAY clusters is shown. The error value of the control pre-mRNAs represents standard deviation of the mean values of 100 random groups of 20 control pre-mRNAs. **c**, YCAY cluster distribution in eight Nova-regulated pre-mRNAs that contain both NISE2 and NISE3 elements.

Effect of Nova binding to a splicing silencer

The RNA map suggests a relationship between the position of YCAY clusters and the mechanism of Nova action. We examined the effects of recombinant Nova1 (Supplementary Fig. 12b) on splicing of two different *in vitro* transcribed RNA substrates (Fig. 3a and Supplementary Fig. 12a) harbouring a YCAY cluster (NESS2 element) within the alternative exon 4 (E4) of Nova1 RNA[20], which has a negative net YCAY score (−1.1, Supplementary Tables 1 and 2). E4 splicing was efficient without Nova, but was inhibited in a dose-dependent manner in the presence of Nova, and this effect was YCAY-dependent (that is, lost when the YCAY motifs were mutated to YAAY; Fig. 3a and Supplementary Fig. 12c, d). These experiments confirm previous data[20], and extend them by demonstrating that Nova directly binds to YCAY clusters to regulate splicing according to the rules of an RNA map.

We analysed the effects of Nova on the stepwise formation of the complexes that lead to spliceosome assembly. Nova inhibits formation of complex E, which results from association of U1 small nucleolar ribonucleoprotein (snRNP) and accessory factors that commit pre-mRNA to splicing[23], and this inhibition required intact YCAY clusters (Fig. 3b). Nova also affected the rate of migration of the early pre-spliceosomal complex H in a YCAY-dependent manner (Fig. 3b), which may result from changes in the ribonucleoprotein complex composition or conformation[24]. To explore whether Nova competes with other proteins assembling on complex H, which itself can be crucial for splicing regulation[25], we performed electrophoretic mobility shift assays using nuclear extracts and Nova1 pre-mRNA substrate (I-E_{YCAY}-I, where I stands for intron, and E for exon). Two shifted bands migrated in a tighter doublet in the presence of Nova (Fig. 3c), and ultraviolet crosslinking studies identified one major protein for which binding was inhibited by Nova1 (Supplementary Fig. 12e). This protein, identified using biotinylated I-E_{YCAY}-I RNA and mass spectrometry of captured proteins, is hnRNP K (Fig. 3d). Comparing the effects of Nova and hnRNP K on E4 splicing using a transfected minigene harbouring the I-E_{YCAY}-I element[20] revealed that Nova had an eightfold greater effect on splicing than hnRNP K (Fig. 3e; see also Supplementary Fig. 12f). These observations suggest that Nova interacts with pre-mRNA before snRNP binding to alter the composition of the pre-spliceosome complex.

Nova can inhibit splicing by blocking U1 snRNP binding

The ability of Nova to interfere with complex E formation suggests that Nova might inhibit access of U1 snRNP to the 5′ splice site. We directly tested the efficiency of U1 snRNP recognition of the 5′ splice site by crosslinking U1 snRNA to I-E_{YCAY}-I RNA[26]. A single crosslinked product was detected in the presence of nuclear extract and found to include U1 snRNP (Fig. 3f, lane 3 versus lane 5). Addition of Nova decreased the formation of the U1–RNA complex 2.5-fold in a YCAY-dependent manner (Fig. 3f, lane 4 versus lane 9, Supplementary Fig. 12g). Thus, Nova binding to the NESS2 YCAY cluster hinders U1 snRNP recognition of the 5′ splice site.

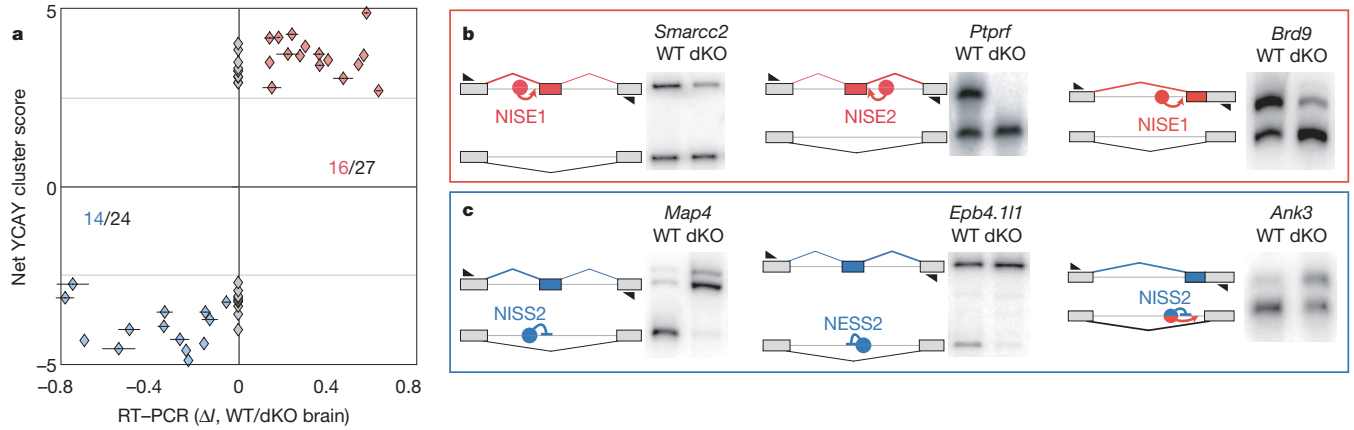

Figure 2 | YCAY cluster position predicts Nova-dependent splicing regulation. **a**, A diagram comparing the bioinformatic prediction of 51 alternative exons having a |net YCAY cluster score| ≥ 2.7 (*y* axis, $n = 3$, error bars indicate s.d.) with change in the fraction of exon inclusion (ΔI)[22] as determined by RT–PCR analysis of wild-type (WT) and *Nova1*$^{-/-}$ *Nova2*$^{-/-}$ (double knockout, dKO) mouse brain (*x* axis). Each RT–PCR experiment was done in biological triplicate (see http://splicing.rockefeller.edu/map). **b**, **c**, Examples of Nova-dependent inclusion (**b**) or skipping (**c**) of alternative exons or splice sites. In the *Ank3* gene, the same element is in a position to act as an NISS2 on the proximal splice site and an NISE1 on the distal splice site. Colour-coded objects present on the left-hand side of each gel represent the pre-mRNA; black triangles indicate the position of the primers used for RT–PCR; circles represent Nova bound to YCAY clusters; arrows indicate enhanced splice sites; bars indicate silenced splice sites.

We also analysed the effect of Nova on spliceosome assembly after complex E formation. Nova entirely inhibited formation of complexes B and C, with most RNA retained in complex H (Fig. 3g, lane 1 versus lane 2; see also Supplementary Fig. 12h). In the presence of Nova a complex co-migrating with complex A was observed, albeit at a slightly reduced level, which seems to conflict with the observation that Nova inhibits complex E formation (which contains U1 snRNP and precedes the formation of complex A). Although complex A normally contains U1 and U2 snRNPs, atypical complexes have been reported to form on substrates containing only a branch site and polypyrimidine tract and in extracts depleted of U1 snRNP[27,28], which agrees with our observation that the band co-migrating with complex A is unaffected by depletion of U1 snRNP but is abolished by depletion of U2 snRNP (Fig. 3g; see also Supplementary Fig. 12i, j). The precise mechanism by which Nova blocks U1 snRNP binding is unknown, but it may involve changes in pre-mRNA secondary structure[29] (Supplementary Fig. 12i). Although Nova did not affect complex A formation in a U1-depleted extract (Fig. 3g, lane 4), the Nova-dependent change in complex H migration occurred in the absence of either U1 or U2 snRNP (Fig. 3g, lanes 4 and 6). These results are consistent with Nova acting locally to change the composition of the pre-spliceosome complex, thereby interfering with the binding of U1 (but not U2) snRNP, and inhibiting exon inclusion.

Effect of Nova binding to a splicing enhancer

To explore the mechanism by which Nova acts on an intronic YCAY cluster, we assayed *in vitro* splicing of a substrate (E-I-E-I_{YCAY}-E RNA), modelled after $GABA_A$ (γ-aminobutyric acid) receptor γ2L pre-mRNA[19] (positive net YCAY score = 3.2; Supplementary Tables 1 and 2), that harboured a YCAY cluster in the middle of a short intron at a position analogous to NISE2 and NISE3 elements. Nova reproducibly mediated a >2-fold increase in the ratio of exon included to skipped product and an accumulation of the second intron lariat; this effect was YCAY-dependent (Fig. 4a; see also Supplementary Fig. 13a–c). When we analysed Nova's effect on spliceosome assembly using an RNA substrate in which the first two exons were joined (EE-I_{YCAY}-E; Fig. 4b) to ensure that the transcript assembled into a single spliceosome, Nova again mediated an increase in the spliced product (1.7-fold, $n = 4$, $P < 1.2 \times 10^{-3}$, two-tailed *t*-test, unequal variance; Supplementary Fig. 13). This effect correlated with increased formation of complex A, followed by complexes B and C (Fig. 4c, d; 1.6-fold, $n = 4$, $P < 0.04$); additional studies suggest that Nova might act on this substrate to affect RNA folding (Supplementary Fig. 13e). These results demonstrate that Nova action at a NISE YCAY cluster promotes spliceosome assembly.

Prediction of asymmetric Nova action on intron removal

Our *in vitro* analysis of Nova-dependent inhibition of exon inclusion suggested that Nova acts locally to affect spliceosome assembly, as Nova inhibited U1 snRNP binding to the E_{YCAY}-I-E RNA 5′ splice site (where the YCAY cluster is located) but not U2 snRNP binding to the 3′ splice site of the same intron (Fig. 3f, g; see also Supplementary Fig. 12i, j). To relate this observation to Nova-dependent splicing regulation in mouse brain, we assessed Nova's effect on splicing of the two introns flanking the alternative exons. We measured RNA splicing intermediates (with one intron unspliced and the other exon–exon junction fully spliced) in 30 exons containing or flanked by Nova silencer or enhancer elements, comparing RNA from *Nova1*$^{-/-}$ *Nova2*$^{-/-}$ relative to wild-type mouse brain (Fig. 5; see also Supplementary Fig. 14).

We tested 16 transcripts in which Nova enhanced exon inclusion via YCAY clusters in the downstream intron (NISE2 or NISE3) and found that Nova was associated with a 5- to 50-fold increase in the 3′ splicing intermediate lacking the intron 3′ of the alternative exon (Fig. 5b; see also Supplementary Fig. 14b). In contrast, Nova was rarely associated with any change in the 5′ splicing intermediate lacking the intron 5′ of the alternative exon, and when it was, the exons were short (~25 nucleotides) and the effects smaller than on the intron with YCAY clusters (Supplementary Table 3). We also tested two transcripts containing YCAY clusters in the intron upstream of the alternative exon (NISE1), and found that in one case (Ddr1, Fig. 5a; see also Supplementary Fig. 14a), Nova splicing enhancement was associated with an asymmetric increase (fourfold) in the 5′, but not 3′, splicing intermediate. Taken together, we detected a significant Nova-dependent increase ($P < 0.05$, two-tailed *t*-test, unequal variance) in at least one splicing intermediate in 17 out of 18 transcripts where Nova promoted alternative exon inclusion. In 12 of these, a significant increase was evident only in a single splicing intermediate, corresponding in each case to an asymmetric action on splicing of the intron harbouring the YCAY cluster (Fig. 5a, b; see also Supplementary Fig. 14a, b and Supplementary Table 3).

We also analysed Nova's effect on splicing intermediates in transcripts where Nova inhibited alternative exon inclusion. Because

Nova's silencing action on splicing intermediates seemed to differ when clusters were located at the 5′ or 3′ end of the alternative exon, we considered separately exonic splicing silencers within 60 nucleotides of either the 3′ splice site (NISS2, NESS1) or the 5′ splice site (NESS2). Nova splicing inhibition was associated with a significant decrease in splicing of the intron harbouring YCAY clusters in 7 out of 7 Nova-regulated exons with upstream clusters and in 4 out of 4 with downstream clusters (NISS2 and NESS1, 2- to 16-fold; NESS2, 2- to 18-fold, respectively, $P < 0.05$, two-tailed *t*-test, unequal variance; Fig. 5c, d, Supplementary Fig. 14d–f and Supplementary Table 3). With the exception of two transcripts containing additional YCAY clusters (Supplementary Fig. 14g), the effect of Nova was limited in each case to one splicing intermediate. Taken together, of the 30 cases tested, 19 had significant ($P < 0.05$) Nova-dependent effects in only one splicing intermediate, and these all showed an asymmetric effect of Nova on splicing of the intron harbouring (or closest to) the YCAY cluster (Supplementary Table 3).

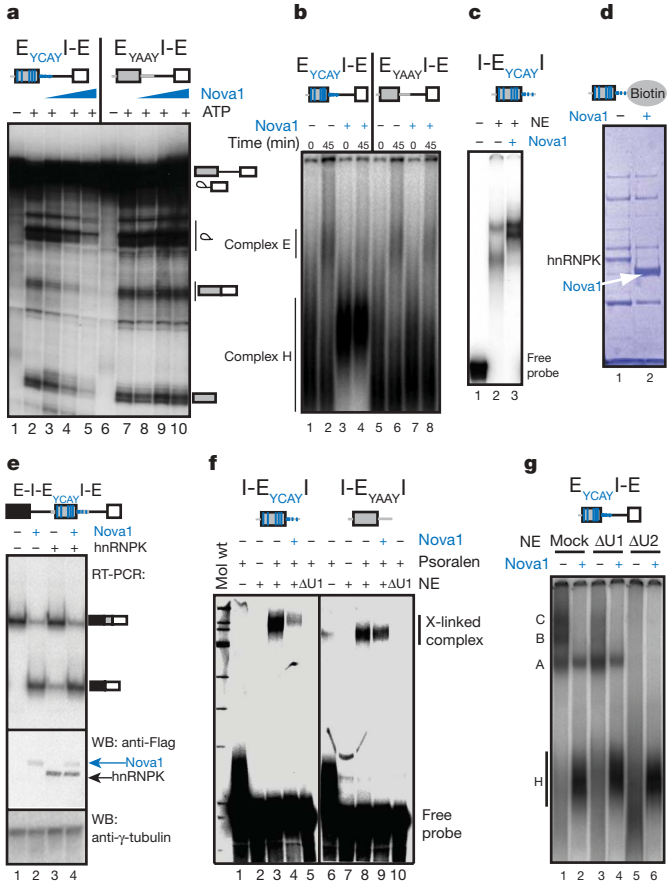

Figure 3 | Nova inhibits splicing of an RNA substrate containing a NESS2 element. **a**, *In vitro* splicing assay using E_{YCAY}-I-E (lanes 1–5) or E_{YAAY}-I-E (lanes 6–10) RNA (1 nM) and nuclear extract in the absence or presence of ATP. Recombinant Nova1 was included in concentrations ranging from 0.03 to 0.12 μM. **b**, Nova inhibits the early phase of spliceosome assembly. A 1 nM concentration of E_{YCAY}-I-E (lanes 1–4) or E_{YAAY}-I-E (lanes 5–8) RNA was incubated on ice or at 30 °C in the absence of ATP. Nova was included at 0.03 μM concentration. **c**, Electrophoretic mobility shift assay. Labelled I-E_{YCAY}-I (1 nM) was incubated in buffer (lane 1) or in HeLa nuclear extract (lane 2). Nova (0.12 μM) was included in lane 3. **d**, RNA affinity purification was performed using 3′-biotinylated I-E_{YCAY}-I from HeLa nuclear extract without (lane 1) or with recombinant Nova (lane 2). Mass spectrometry analysis identified the 58-kDa band as hnRNP K. **e**, Effects of Flag–HA–Nova1 and Flag–hnRNP K overexpression on splicing of empty expression vector (lane 1) or E-I-E_{YCAY}-I-E minigene (lanes 2–4) (Supplementary Fig. 11a). **f**, Psoralen crosslinking assay. Labelled I-E_{YCAY}-I (lanes 1–5) or I-E_{YAAY}-I RNA (lanes 6–10) was incubated at 1 nM concentration in the absence (lane 1 and 6) or presence (lanes 2–5 and 7–10) of HeLa nuclear extract (NE). In lanes 5 and 10, the 5′ end of U1 snRNA was removed by oligodeoxynucleotide-mediated RNase H treatment (ΔU1). Recombinant Nova1 (0.12 μM final concentration) was included in lanes 4 and 9. **g**, Spliceosome assembly in the presence of ATP. Labelled E_{YCAY}-I-E pre-mRNAs (1 nM) were incubated in nuclear extracts (NE) that were mock-depleted (lanes 1 and 2), depleted of U1 snRNP (ΔU1, lanes 3 and 4), or U2 snRNP (lanes 5 and 6). Recombinant Nova (0.03 μM) was included in lanes 2, 4 and 6. Vertical blue lines in all cartoons represent YCAY motifs.

Discussion

We used over 50 previously identified, genetically validated Nova-regulated alternative transcripts[14,20–22] to derive a general RNA map relating the sequence and position of YCAY clusters to Nova activity (Fig. 1). When used to screen the mouse genome, this RNA map correctly predicted Nova splicing silencers and enhancers in another 30 alternatively spliced transcripts (Fig. 2). The predictive accuracy of the map suggested a mechanistic link between the position where Nova binds YCAY clusters and the splicing outcome. Assays in a reconstituted splicing system demonstrated that Nova acts on a NESS2 YCAY cluster to interfere specifically with U1 snRNP binding

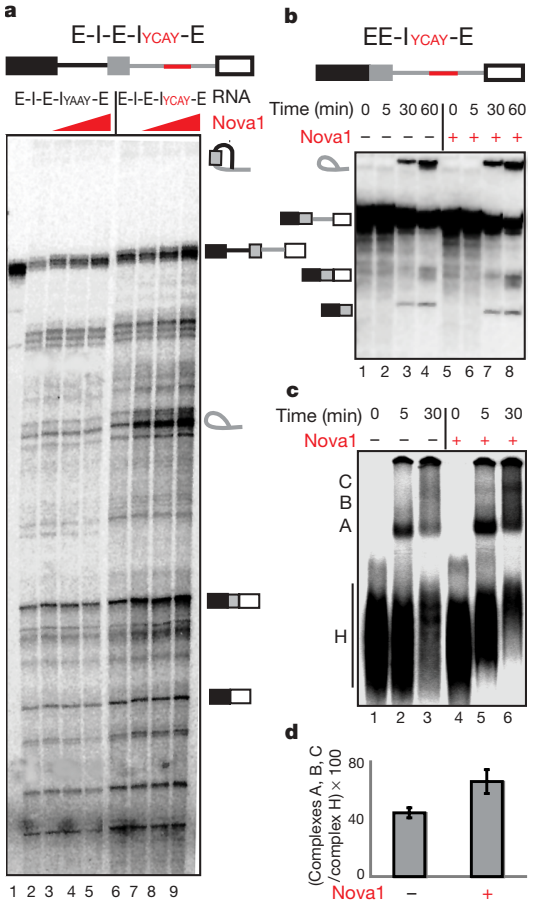

Figure 4 | Nova binding to a splicing enhancer promotes spliceosome assembly. **a**, Labelled E-I-E-I$_{YAAY}$-E (lanes 1–5) or E-I-E-I$_{YCAY}$-E (lanes 6–9) RNA was incubated for 4 h at 30 °C with HeLa nuclear extract in the absence of ATP (lane 1) or in splicing conditions (lanes 2–9) at 1 nM concentration in the absence (lanes 2 and 6) or presence of Nova (concentrations ranging from 0.02 to 0.08 μM, lanes 3–5 and 7–9). **b**, Labelled EE-I$_{YCAY}$-E RNA was incubated in splicing conditions in the absence (lanes 1–4) or presence (lanes 5–8) of 0.12 μM Nova. **c**, Spliceosome assembly in the presence of ATP. Radiolabelled EE-I$_{YCAY}$-E (1 nM) was incubated in nuclear extract (NE) in the absence or presence of recombinant Nova (0.12 μM). **d**, Quantification of the gel shown in **c** ($n = 4$, error bars indicate s.d.).

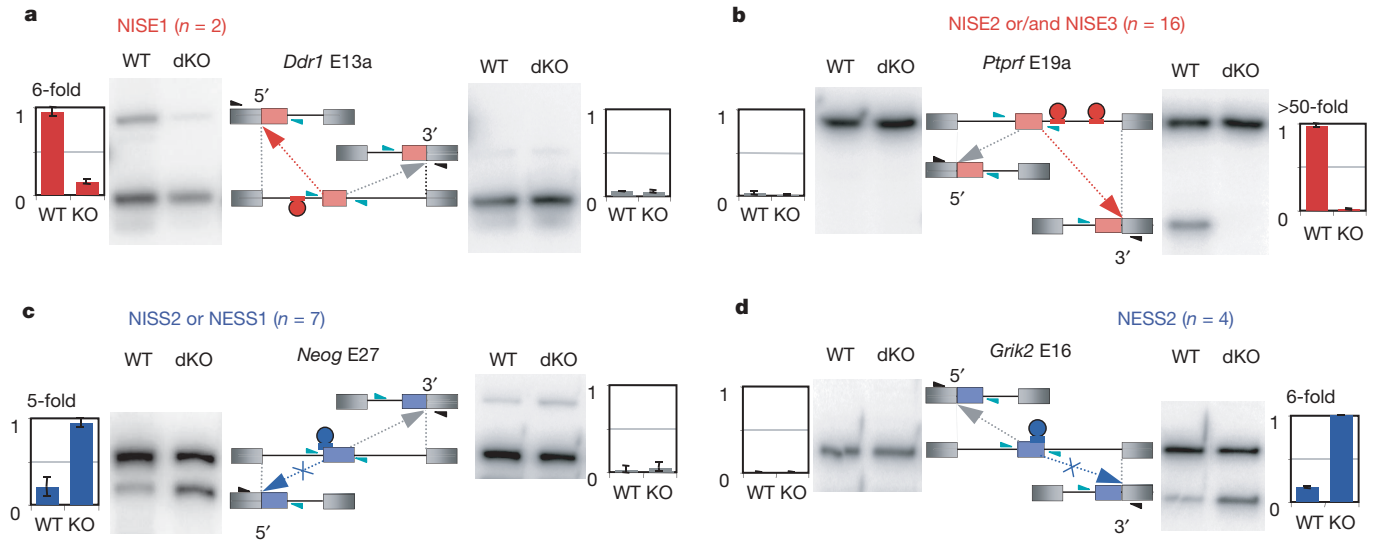

Figure 5 | Analysis of splicing intermediates in Nova-regulated RNAs. Quantification of splicing intermediates that contain one unspliced intron and the other exon–exon junction spliced, using RNA from *Nova1*$^{-/-}$ *Nova2*$^{-/-}$ (dKO) and wild-type littermate brains. In addition to the primer pair that amplifies the splicing intermediate, two additional primers amplifying pre-mRNA were used to monitor the efficiency of all primers (for visual clarity, only one pre-mRNA control band is shown here; both control bands are shown in Supplementary Fig. 14). The graphs show quantification of bands representing splicing intermediates, normalized to the pre-mRNA bands ($n = 3$, error bars indicate s.d.). Because the two splicing intermediates represent two alternative pathways leading to exon inclusion, the added value of bands in wild-type samples was normalized to 1 when Nova promotes exon inclusion, and the added value in *Nova1*$^{-/-}$ *Nova2*$^{-/-}$ double knockout samples was normalized to 1 when Nova promotes exon skipping. Colour-coded objects present represent the pre-mRNA, splicing intermediates, the position of the primers used for RT–PCR (triangles), and position of Nova bound to YCAY clusters (circle). Each RT–PCR experiment was done in biological triplicate and the identity of bands was verified by sequencing, and where PCR products were short enough, quantified by real-time PCR (Supplementary Fig. 14). **a**, Representative of two tested pre-mRNAs containing a NISE1 element. **b**, Representative of 16 tested pre-mRNAs containing NISE2 and/or NISE3 elements. **c**, Representative of seven tested pre-mRNAs containing NESS1 or NISS2 elements. **d**, Representative of four pre-mRNAs containing a NESS2 element.

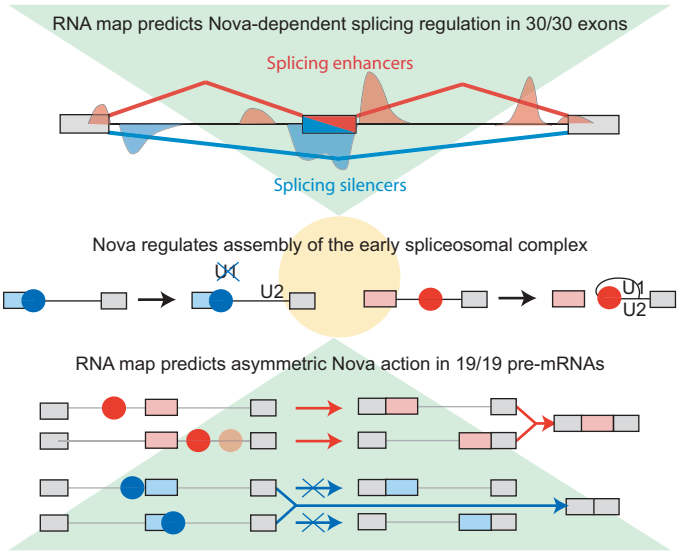

Figure 6 | An RNA map providing a comprehensive view of Nova function. An RNA map was defined using an algorithm that analysed the distribution of Nova binding sites (YCAY clusters)[14–16] in genetically validated Nova-target pre-mRNAs[12,18–20]. The RNA map was applied to a genome-wide screen to predict correctly whether Nova acted to enhance or block alternative exon inclusion in all of the top scoring and validated 30 exons (top triangle). The RNA map relates to the precise mechanism of Nova action to enhance or inhibit spliceosome assembly in the vicinity of YCAY clusters, as determined in a reconstituted system *in vitro* (circle). These results were generalized by quantification of splicing intermediates in Nova knockout brain, which showed that the RNA map predicts the *in vivo* site of Nova action in each of 19 pre-mRNAs where we detected an asymmetry in Nova's action on removal of the two introns flanking the alternative exons (bottom triangle). This data defines a genome-wide RNA map in which the position of *cis*-acting elements determines the outcome of regulation by the splicing factor Nova.

(Fig. 3) and on a NISE2/3 YCAY cluster to promote spliceosome assembly (Fig. 4). Furthermore, quantification of splicing intermediates in brain of Nova knockout (*Nova1*$^{-/-}$ *Nova2*$^{-/-}$) mice demonstrated a direct and asymmetric *in vivo* action of Nova on introns harbouring (or closest to) YCAY binding elements (Fig. 5). These data support a mechanistic model in which the RNA map dictates a local action of Nova to regulate spliceosome assembly (Fig. 6 and Supplementary Fig. 1).

A validated RNA map provides new insights into functional RNA–protein interactions. Defining Nova binding sites as YCAY clusters[16–21] (Supplementary Fig. 3) allowed us to develop a bioinformatic procedure with high predictive accuracy, suggesting more generally that searching for clustered motifs may uncover regulatory elements overlooked in studies focusing on single, short splicing motifs[3,6,7,10]. The degree of YCAY cluster conservation between mouse and human genomes (97%; Supplementary Fig. 3c) agrees with previous bioinformatic estimates that 98% of major form alternative exons are conserved[30]. Nova-regulated targets that did not have high YCAY cluster scores within the region of the current RNA map (Supplementary Fig. 4 and Supplementary Table 1) may be regulated by YCAY clusters located outside of the current RNA map, by Nova-dependent regulation of other splicing factors, by interactions of Nova with other splicing factors, or by other means. Notably, we found an over-represented motif, CACCA, in nine NESS elements with top YCAY cluster scores. This pentamer partially overlaps Nova binding sites (YCACCAY; Supplementary Fig. 9b) and was previously shown to act as an exonic enhancer[31,32], suggesting that in some transcripts Nova may compete or synergize with other factors (such as hnRNP K (Fig. 3) or brPTB/nPTB[33]) to regulate splicing.

Nova's displacement of U1 snRNP by binding to the NESS2 element (Figs 3 and 5c) is reminiscent of the ability of Sxl[34], hnRNP A1[35,36] and PTB[25,37] to displace U2AF$_{65}$ or U2 snRNP, which is how Nova might act when binding NISS2 and NESS1 elements in the vicinity of the 3′ splice site (Fig. 5d). In contrast, Nova binding to

intronic YCAY clusters generally promotes spliceosome assembly. Although splicing enhancers have most often been associated with exonic sequences[3,5,8,31], in some instances proteins such as TIA1[37], KSRP, hnRNP F/H[10,38], Fox1 and Fox2[39] promote spliceosome assembly by binding to intronic sequences downstream of the exon. Notably, Nova-regulated exons flanked by NISE2 elements are generally shorter than 50 nucleotides ($n = 12$, $P = 0.003$; Supplementary Table 3 and Supplementary Fig. 9a), consistent with suggestions that intronic enhancer elements are particularly important in regulating short exons[40], which themselves have a lower chance of harbouring exonic splicing enhancers. Of the 13 Nova-target pre-mRNAs with NISE3 elements (near the downstream constitutive exon), 10 also contain a NISE2 element (near the alternative exon; Fig. 1c and Supplementary Tables 1 and 3), suggesting that Nova might bind both sites and multimerize[41] to form an RNA loop bringing the 5′ splice site and the branch site into close proximity. Models of splicing regulation by factors involved in RNA looping or binding to multiple sites have been proposed for brPTB/nPTB[42], hnRNP A/B[36,43] and hnRNP F/H[43].

We quantified partially spliced RNAs in the brain of *Nova1*$^{-/-}$ *Nova2*$^{-/-}$ mice, demonstrating that Nova in most cases (19 out of 30) acts asymmetrically to regulate splicing of only one intron flanking the alternative exon. In all of these cases (19 out of 19) the regulated intron contains (or is proximal to) YCAY clusters (Fig. 6 and Supplementary Fig. 1). These observations support a model in which local, asymmetric actions of Nova enhance splicing of the 3′ and 5′ intron via NISE1 and NISE2/NISE3, respectively, and silence splicing of the 3′ and 5′ intron via NISS2/NESS1 and NESS2, respectively.

How can exon skipping be induced by Nova if it only prevents splicing of one intron flanking the alternative exon, while splicing of the other intron remains equally efficient? Intuitively, exon skipping should require repression of both splicing events to generate a product in which the exon is excluded. However, pre-mRNAs containing exons that are silenced in the presence of Nova most often display some degree of asymmetry of splicing intermediates even in the absence of Nova, suggesting that splicing of one of the introns represents the rate limiting step for exon inclusion (Fig. 5c, d; see also Supplementary Fig. 14c–e). The mechanism underlying this predetermined asymmetry is unclear, because it does not correlate with splice site scores of Nova-regulated exons, which on average have consensus splice site sequences similar to those of constitutive exons (Supplementary Fig. 10). Notably, in all transcripts with such predetermined asymmetry, the Nova silencer elements are located at the intron that is normally removed first, thus enabling Nova to inhibit the rate-limiting step for exon inclusion (Fig. 5c, d; see also Supplementary Fig. 14c–e). Our data support previous biochemical studies indicating that pre-mRNAs often follow a preferential splicing order[44–47], and suggest that such an order may allow proteins like Nova to change splicing outcome by locally regulating the rate limiting step leading to alternative exon inclusion.

This study identified 23 new Nova-regulated exons (Supplementary Table 3), 12 of which encode proteins with known functions in brain. Eleven out of 12 of these proteins act in adhesion, cytoskeleton binding, neuronal inhibition, signalling, or synapse formation and plasticity (http://splicing.rockefeller.edu/map/validated). These observations agree with and extend previous findings that Nova, as well as other regulators of gene expression, tend to co-regulate genes encoding functionally related proteins[21,22,48] (see ref. 11 for a review). For example, previous microarray analysis had identified *Ptprf* exon 19a, *Ank3* exon 17 and *Epb4.1* exon 14 (ref. 22), and the current study identified *Ptprf* exon 6a, *Ank3* exon 31 and *Epb4.1l1* exon 16 as Nova targets; in addition, two Nova-regulated exons encode variants of Dab1 (Supplementary Figs 6c, 7a), a protein known to participate in the pathway of reelin-dependent synaptic plasticity modulation[49]. It remains to be tested whether alternative splicing of these and other Nova-regulated exons contributes to long term potentiation of slow inhibitory postsynaptic currents, which is defective in *Nova2*$^{-/-}$ hippocampus[50], or other aspects of neuronal physiology.

Our analysis of functional Nova–RNA interactions leads to several new conclusions (Fig. 6 and Supplementary Fig. 1). We provide an RNA map predicting the activity of a splicing regulator in which splicing silencers differ from enhancers in their position on the pre-mRNA, but share a similar sequence. Whereas sequences close to alternative exons have been the focus of previous bioinformatic studies[3–10], we find that sequences close to the flanking constitutive exons have equally important roles in the regulation of alternative splicing. Finally, our analysis provides a new model whereby Nova regulates exon inclusion through a direct and asymmetric action on one of the flanking introns, by locally blocking or enhancing assembly of the spliceosome. As a general approach, our data suggest that a detailed understanding of the nature of protein–RNA interactions, together with identification of a large set of RNA targets validated in a genetic system, can be combined with bioinformatics and biochemistry to identify the sites and mechanisms of action of alternative splicing factors.

METHODS

Pre-mRNAs containing 48 exons that were previously found to be Nova-regulated[14,20–22] (excluding special cases, such as intron retention or redundant consecutive exons, Supplementary Tables 1 and 2) were analysed to optimize the procedure for calculating the YCAY cluster score (Supplementary Fig. 3; see also Supplementary Methods). The YCAY cluster score was quantified using three criteria: the number of YCAY motifs, the distance between YCAY motifs, and the degree of evolutionary conservation, using an algorithm that maximized the accuracy and efficiency of predicting Nova-regulated, but not control exons (Supplementary Fig. 4). Net YCAY cluster score was calculated by subtracting the maximum score of NISS and NESS elements from the maximum score of NISE elements, such that a positive net YCAY cluster score predicts that Nova promotes exon inclusion, and a negative score predicts that Nova promotes exon skipping. Fifty-one candidate alternative exons had a predicted |net YCAY cluster score| > 2.7, and we tested splicing of these in *Nova1*$^{-/-}$ *Nova2*$^{-/-}$ embryonic day 18 mouse brain by polymerase chain reaction with reverse transcription (RT–PCR). All 51 predicted alternative exon candidates were successfully detected and confirmed by sequencing (see http://splicing.rockefeller.edu/map); it is uncertain whether our ability to predict alternative exons and genes that are expressed at day 18 embryonic brain relates to our stringent filtering for alternative exons, the presence of YCAY clusters, or other variables.

Recombinant Nova1 (Supplementary Fig. 12b) was used for all *in vitro* assays, described in Supplementary Methods. The substrates used for *in vitro* assays were E-I-E$_{YCAY}$-I-E and its short versions (E$_{YCAY}$-I-E and I-E$_{YCAY}$-I), and E-I-E-I$_{YCAY}$-E and its short version E-I$_{YCAY}$-E. Analysis of splicing intermediates by RT–PCR was performed using RNA from *Nova1*$^{-/-}$ *Nova2*$^{-/-}$ embryonic day 18 mouse brain and quantitative autoradiography or real-time PCR.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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