

spreading might entail disruption of complexes, with only some subunits actually involved in spreading. In this context, it is interesting to note that, on active X-linked genes, the Male absent on the first (MOF) acetyltransferase does not colocalize perfectly with MSL3. Instead, MSL3 binding is biased toward the 3' end of genes, whereas MOF shows a bimodal distribution (enriched at both the promoter and the 3' end)<sup>19</sup>. Therefore it will be important to determine whether MOF binding is dependent on the chromodomain of MSL3. Adding to the complexity of the spreading mechanism is the finding that MOF and MSL1 (Fig. 2) are required for MSL3 binding to low-affinity sites<sup>19</sup>. This indicates that there might be additional mechanisms that regulate MSL3 binding to H3K36me3.

Regardless of the spreading mechanism, the Sural *et al.* paper<sup>7</sup> adds to recent studies that have revealed surprising parallels between repressive and active chromatin domains. First, formation of heterochromatin requires the active transcription of noncoding RNA. In addition, proteins (such as HP1) and histone marks (such as H3K9me) that were once thought to be unique to repressive chromatin have since been found within coding regions of active genes<sup>20,21</sup>. Therefore, it seems that mechanisms used to generate repressive heterochromatin might be conserved, at least in part, for the formation of large domains

of active chromatin. Clearly, spreading does not occur at every locus, but it might be particularly important for coordinated regulation of clustered genes such as Hox or  $\beta$ -globin genes. Interestingly, spreading of the H3K4 methyltransferases MLL1 and MLL2 have been observed at these loci<sup>22,23</sup>. Although MLL spreading could be mediated via interaction with the elongating polymerase<sup>24</sup>, the study by Sural *et al.* suggests that it could also entail interaction with methylated histones. Indeed, the MLL family of proteins contains PHD domains that can recognize specific methyl marks. In fact, proteins containing domains of recognition for specific histone modifications, including bromo- and chromodomains, WD40 repeats or ankyrin domains<sup>25,26</sup>, are all potential candidates to mediate complex spreading.

Although many questions remain about the exact molecular mechanism of spreading, as well as its role in coordinating gene expression, Sural *et al.* have paved the way for future discoveries related to the structure and function of large domains of active chromatin.

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- Li, B., Carey, M. & Workman, J.L. *Cell* **128**, 707–719 (2007).
- Talbert, P.B. & Henikoff, S. *Nat. Rev. Genet.* **7**, 793–803 (2006).
- Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. & Jenuwein, T. *Nature* **410**, 116–120 (2001).
- Elgin, S.C. & Grewal, S.I. *Curr. Biol.* **13**, R895–R898 (2003).
- Bulger, M. *J. Biol. Chem.* **280**, 21689–21692 (2005).
- Lucchesi, J.C., Kelly, W.G. & Panning, B. *Annu. Rev. Genet.* **39**, 615–651 (2005).
- Sural, T.H. *et al. Nat. Struct. Mol. Biol.* **15**, 1318–1325 (2008).
- Buscaino, A., Legube, G. & Akhtar, A. *EMBO Rep.* **7**, 531–538 (2006).
- Krogan, N.J. *et al. Mol. Cell. Biol.* **23**, 4207–4218 (2003).
- Straub, T. & Becker, P.B. *Curr. Opin. Genet. Dev.* **18**, 175–180 (2008).
- Kelley, R.L. *et al. Cell* **98**, 513–522 (1999).
- Kelley, R.L. *Dev. Biol.* **269**, 18–25 (2004).
- Alekseyenko, A.A. *et al. Cell* **134**, 599–609 (2008).
- Gilfillan, G.D. *et al. Nucleic Acids Res.* **35**, 3561–3572 (2007).
- Kind, J. & Akhtar, A. *Genes Dev.* **21**, 2030–2040 (2007).
- Larschan, E. *et al. Mol. Cell* **28**, 121–133 (2007).
- Bell, O. *et al. Mol. Cell. Biol.* **28**, 3401–3409 (2008).
- Schwartz, Y.B. & Pirrotta, V. *Nat. Rev. Genet.* **8**, 9–22 (2007).
- Kind, J. *et al. Cell* **133**, 813–828 (2008).
- Vakoc, C.R., Mandat, S.A., Olenchok, B.A. & Blobel, G.A. *Mol. Cell* **19**, 381–391 (2005).
- Huisinga, K.L., Brower-Toland, B. & Elgin, S.C. *Chromosoma* **115**, 110–122 (2006).
- Guenther, M.G. *et al. Proc. Natl. Acad. Sci. USA* **102**, 8603–8608 (2005).
- Demers, C. *et al. Mol. Cell* **27**, 573–584 (2007).
- Eissenberg, J.C. & Shilatifard, A. *Curr. Opin. Genet. Dev.* **16**, 184–190 (2006).
- Taverna, S.D., Li, H., Ruthenburg, A.J., Allis, C.D. & Patel, D.J. *Nat. Struct. Mol. Biol.* **14**, 1025–1040 (2007).
- Collins, R.E. *et al. Nat. Struct. Mol. Biol.* **15**, 245–250 (2008).

## Alternative splicing: regulation without regulators

Brenton R Graveley

Alternative splicing is typically thought to be controlled by RNA binding proteins that modulate the activity of the spliceosome. A new study not only demonstrates that alternative splicing can be regulated without the involvement of auxiliary splicing factors, but also provides mechanistic insight into how this can occur.

The field of alternative splicing is moving forward at a breathtaking pace. Only a little more than 10 years ago, it was thought that most human genes encoded only a single mRNA isoform. However, it is now known that alternative splicing is the rule, not the

exception—~90% of human genes encode at least two isoforms, and most known alternative-splicing events are regulated<sup>1,2</sup>. This implies that there is an extensive network of splicing regulators to control the plethora of alternative-splicing events. However, the number of known splicing regulators (<50) and even the number of known RNA binding proteins encoded by the human genome (<300) cannot alone be responsible for controlling all of the alternative-splicing events we know about. Are there other types of RNA binding proteins that we are unaware of? Are there mechanisms for controlling alternative splicing that fall outside our traditional view?

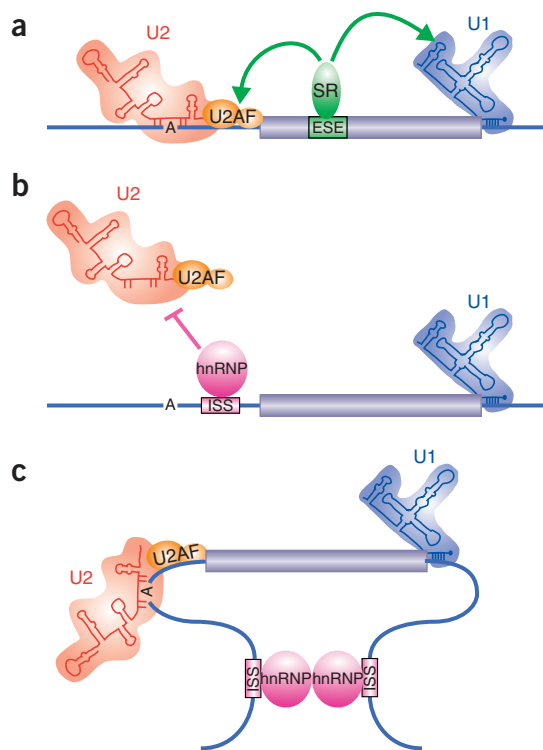
Although the answers to both questions are likely to be yes, a recent paper in *Cell* by Nilsen and colleagues<sup>3</sup>, provides strong evidence for the latter—alternative splicing can be controlled in a manner that is independent of known splicing regulators.

Traditional models of regulated alternative splicing involve auxiliary splicing factors—proteins that are not core components of the spliceosome, but rather bind to the pre-mRNA and either enhance or repress the ability of the spliceosome to recognize particular splice sites. Two important classes of auxiliary splicing factors are SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNP) proteins.

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SR proteins typically bind to sequences within exons called exonic splicing enhancers (ESEs), where they interact with and recruit various components of the spliceosome to adjacent splice sites<sup>4</sup>. For example, SR proteins can enhance 3' splice site recognition by recruiting U2AF to the upstream polypyrimidine tract and can enhance 5' splice site recognition by interacting with U1-70K and recruiting the U1 small nuclear RNP (snRNP) to the downstream 5' splice site (Fig. 1a). The hnRNP proteins, in contrast, typically bind to exonic splicing silencers (ESSs) or intronic splicing silencers (ISSs) and repress splicing by various mechanisms<sup>5</sup>. hnRNP proteins can repress splicing by steric hinderance—for instance, by binding to the polypyrimidine tract and preventing U2AF binding (Fig. 1b)—or by sequestration, for example, by binding to ISSs in the introns flanking an exon and looping the exon out of the pre-mRNA (Fig. 1c). Although there are several examples of how splicing enhancers and silencers function, it is clear that much remains to be learned about how this important aspect of gene regulation is controlled.

To explore further the mechanisms by which splicing silencers function, Yu *et al.*<sup>3</sup> set out first to identify *cis*-acting RNA sequences that can function as splicing silencers, and then to determine the mechanisms by which these silencers function. The authors began by designing a synthetic splicing substrate containing two competing 5' splice sites and a single 3' splice site (Fig. 2a). The upstream 5' splice site was designed to be weak so that it was used inefficiently, whereas the downstream 5' splice site was designed to be strong. As a result, the strong downstream 5' splice site was predominantly used when this pre-mRNA was spliced *in vitro*. The authors then inserted randomized sequences in either the exon or intron flanking the strong 5' splice site and performed several rounds of selection experiments to enrich for sequences that silenced the strong 5' splice site and promoted use of the weak 5' splice site (Fig. 2b). They also used a clever counterselection step to remove sequences that act by preventing U1 snRNP from binding to the strong 5' splice site. Bioinformatic analysis of ~150 active sequences yielded two exonic and four intronic groups of related sequences that silenced the strong 5' splice site in both *in vitro* biochemical assays and in tissue-culture cells. Further analysis revealed that four of the isolated sequence motifs were similar to motifs previously shown to be enriched near pseudo-5' splice sites<sup>6–9</sup>—sequences that look like genuine 5' splice sites but are not used by the spliceosome. This suggests



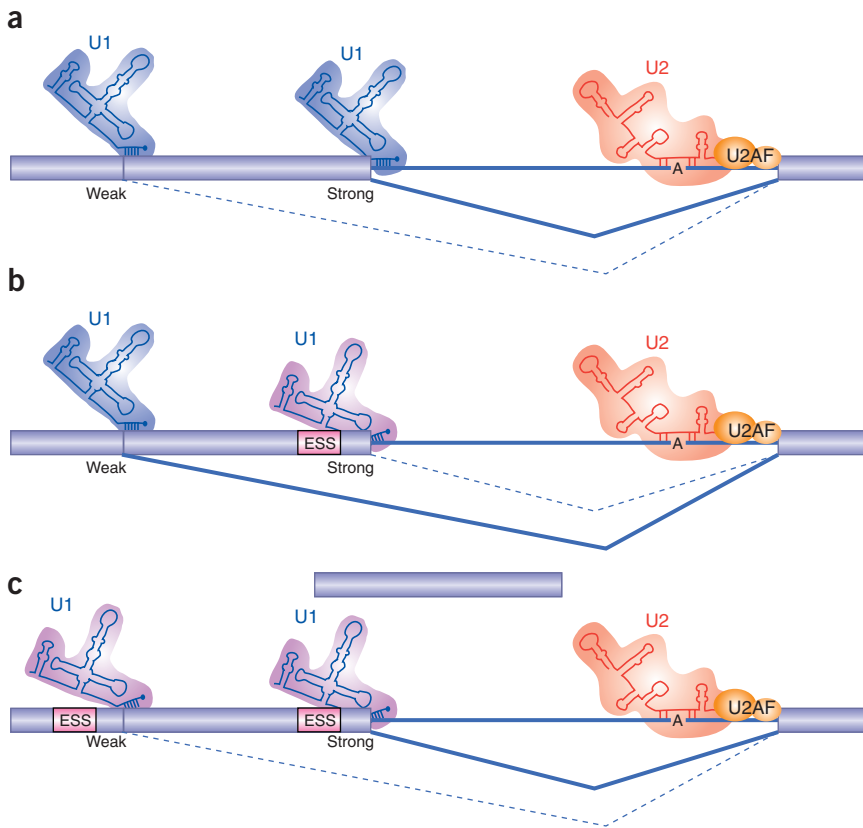
**Figure 1** Traditional splicing regulation. (a) Splicing activators such as SR proteins can function by binding to exonic splicing enhancers (ESEs), where they interact with and recruit U2AF and/or U1 snRNP to the 3' and 5' splice sites, respectively. (b) Splicing repressors such as hnRNP proteins can inhibit splicing by binding to intronic splicing silencers (ISSs), where they interfere with the binding of U2AF to the 3' splice site. (c) Alternatively, hnRNP proteins can bind to ISSs in the introns flanking an exon, where they engage in protein-protein interactions and loop out the intervening exon.

that the motifs isolated in these experiments are likely to function as authentic splicing silencers throughout the human genome.

The story took a very interesting turn when Yu *et al.*<sup>3</sup> focused their attention on determining the mechanism by which the silencers function. The first indication of something interesting was the observation that the silencers repressed only the 5' splice site in pre-mRNAs containing competing 5' splice sites—the silencer had little impact on the strong 5' splice site in pre-mRNAs lacking the weak 5' splice site. Second, pre-mRNAs containing silencers near both 5' splice sites predominantly used the strong 5' splice site, just like pre-mRNAs that lacked the silencer altogether (Fig. 2c). These two observations strongly suggested that the silencers do not impair the ability of U1 snRNP to recognize the 5' splice site, but rather alter the efficiency with which the U1 snRNP–5' splice site complex can engage in splicing. Consistent with this notion, Yu *et al.* found that, although the silencers did not impact the ability of U1 snRNP to bind to the 5' splice site, they did alter the manner in which U1 snRNP interacted with the 5' splice site both in nuclear extracts and with highly purified U1 snRNP.

These observations, and the fact that extensive searches failed to identify any proteins that specifically recognize the silencers, suggest that sequences at the 5' splice sites can strongly influence the efficiency with which the splice sites can be used in a manner that does not require auxiliary splicing regulators.

How can these findings be placed in the context of the field? Although the vast majority of work on alternative splicing has focused on hnRNP, SR and related proteins and the mechanisms by which they act, there have been scattered indications that alternative splicing can be regulated by mechanisms that do not involve auxiliary splicing regulators. For example, an RNA interference screen in *Drosophila melanogaster* tissue-culture cells found that individually depleting components of the spliceosome can have a dramatic impact on the alternative splicing of specific pre-mRNAs<sup>10</sup>. Similarly, studies in *Saccharomyces cerevisiae* have shown that perturbing the activity of various spliceosomal components results in robust changes in the efficiency of intron removal for distinct pre-mRNAs<sup>11</sup>. Moreover, it is now known that several aspects of the pre-mRNA splicing reaction can proceed



**Figure 2** Regulation without regulators. (a) In the absence of a splicing silencer, the strong downstream 5' splice site is used more efficiently than the weak upstream 5' splice site, despite the fact that U1 snRNP binds to both 5' splice sites. (b) When a splicing silencer (ESS) is present in the exon near the strong downstream 5' splice site, use of the upstream weak 5' splice site now predominates. Importantly, the ESS does not prevent U1 snRNP from binding to the strong 5' splice site, although the configuration in which U1 snRNP interacts is altered. (c) When the ESS is present near both 5' splice sites, it alters the association of U1 snRNP with both 5' splice sites in the same way. As a result, efficient splicing to the strong downstream 5' splice site is restored.

in both the forward and reverse directions, including the catalytic steps<sup>12,13</sup>. Together, these observations, along with the recent results from Yu *et al.*<sup>3</sup>, all suggest that modulations in the activity or concentration of general splicing factors would alter the kinetic equilibrium

of one or more steps in the splicing reaction, resulting in changes in splice-site selection.

In conclusion, it is now clear that alternative splicing can, at least in some cases, be regulated by mechanisms that do not involve auxiliary splicing regulators. The results of Yu *et al.*<sup>3</sup>

provide the first glimpse at the types of mechanisms that could account for this unconventional mode of splicing regulation. Although these new findings are quite exciting, they also raise many important questions. How many alternative-splicing events are regulated without the aid of auxiliary splicing regulators? Which spliceosomal components and RNA sequence elements are involved in this type of regulation? Which steps in spliceosome assembly and catalysis are subject to regulation? Initial answers to these questions are certain to come from genome-wide screens in which numerous splicing factors are perturbed. Such studies will identify alternative-splicing events that are regulated by specific components of the splicing machinery. This information will set the stage for determining the myriad mechanisms of regulator-independent alternative splicing. Although there is clearly much work ahead, Nilsen and colleagues have made a significant advancement in our understanding of the intricacies of alternative-splicing regulation.

1. Pan, Q., Shai, O., Lee, L.J., Frey, B.J. & Blencowe, B.J. *Nat. Genet.* **40**, 1413–1415 (2008).
2. Wang, E.T. *et al.* *Nature* **456**, 470–476 (2008).
3. Yu, Y. *et al.*, *Cell* published online, doi:10.1016/j.cell.2008.10.046 (26 December 2008).
4. Lin, S. & Fu, X.D. in *Alternative Splicing in the Postgenomic Era* (eds. Blencowe, B. J. & Graveley, B. R.) 107–122 (Landes Bioscience and Springer Science + Business Media, LLC, New York, NY, 2007).
5. Martinez-Contreras, R. *et al.* in *Alternative Splicing in the Postgenomic Era* (eds. Blencowe, B. J. & Graveley, B. R.) 123–147 (Landes Bioscience and Springer Science+Business Media, LLC, New York, 2007).
6. Wang, Z. *et al.* *Cell* **119**, 831–845 (2004).
7. Wang, Z., Xiao, X., Van Nostrand, E. & Burge, C.B. *Mol. Cell* **23**, 61–70 (2006).
8. Zhang, X.H. & Chasin, L.A. *Genes Dev.* **18**, 1241–1250 (2004).
9. Zhang, X.H., Heller, K.A., Heffer, I., Leslie, C.S. & Chasin, L.A. *Genome Res.* **13**, 2637–2650 (2003).
10. Park, J.W., Parisky, K., Celotto, A.M., Reenan, R.A. & Graveley, B.R. *Proc. Natl. Acad. Sci. USA* **101**, 15974–15979 (2004).
11. Pleiss, J.A., Whitworth, G.B., Bergkessel, M. & Guthrie, C. *PLoS Biol.* **5**, e90 (2007).
12. Smith, D.J., Query, C.C. & Konarska, M.M. *Mol. Cell* **30**, 657–666 (2008).
13. Tseng, C.K. & Cheng, S.C. *Science* **320**, 1782–1784 (2008).