Shiotani and Zou's work provides crucial mechanistic insight into the emerging consensus on the dynamic recognition of double-strand breaks. In addition, it holds the promise of further biochemical dissection of the complicated interplay between proteins at the ends of broken DNA.

REFERENCES

Choi, J.H., Lindsey-Boltz, L.A., and Sancar, A. (2007). Proc. Natl. Acad. Sci. USA *104*, 13301–13306.

Dupre, A., Boyer-Chatenet, L., and Gautier, J. (2006). Nat. Struct. Mol. Biol. *13*, 451–457.

Gravel, S., Chapman, J.R., Magill, C., and Jackson, S.P. (2008). Genes Dev. *22*, 2767–2772.

Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G.C., Lukas, J., and Jackson, S.P. (2006). Nat. Cell Biol. *8*, 37–45.

Lee, J.H., and Paull, T.T. (2005). Science *308*, 551–554.

MacDougall, C.A., Byun, T.S., Van, C., Yee, M.C., and Cimprich, K.A. (2007). Genes Dev. *21*, 898–903.

Mimitou, E.P., and Symington, L.S. (2008). Nature *455*, 770–774.

Myers, J.S., and Cortez, D. (2006). J. Biol. Chem. *281*, 9346–9350.

Shiotani, B., and Zou, L. (2009). Mol. Cell *33*, 547–558.

Yoo, H.Y., Kumagai, A., Shevchenko, A., Shevchenko, A., and Dunphy, W.G. (2007). J. Biol. Chem. *282*, 17501–17506.

Zhu, Z., Chung, W.H., Shim, E.Y., Lee, S.E., and Ira, G. (2008). Cell *134*, 981–994.

Getting under the Skin of Alternative Splicing: Identification of Epithelial-Specific Splicing Factors

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Alternative splicing patterns are regulated by factors that direct the activity of the spliceosome. In a recent issue of *Molecular Cell*, Warzecha et al. (2009) identify two new splicing regulators whose epithelial-specific expression induces several tissue-specific splicing events.

Alternative splicing is a prominent feature of eukaryotic genomes. The differential inclusion or skipping of variable exons occurs in the majority of human genes and is a major contributor to proteome diversity and regulation of gene expression (Matlin et al., 2005). Moreover, recent technical advances such as splicing-sensitive microarrays and deep sequencing have identified distinct tissue-specific splicing patterns on a genome-wide scale (Pan et al., 2008; Wang et al., 2008). In many cases, the genes that show coregulation at the level of splicing in a particular tissue cluster into physiologically meaningful ontologies (Ule et al., 2005); however, with few exceptions, the functional significance of this coregulation has not been formally shown. Furthermore, there is little understanding of how such cell-type-specific splicing profiles are established. While the weight of current evidence argues against tissue-specific "master-regulators" that act alone to dictate splicing patterns, it is unclear whether tissue-specific splicing is due to the differential fine-tuning of many ubiquitously expressed splicing factors, to the presence of a few dominant determinant proteins, or to somewhere in between (Matlin et al., 2005; Figure 1).

A handful of cell-type-specific splicing factors have been identified in the past decade; however, these have been limited to proteins that specify neural- or muscle-specific splicing events (Li et al., 2007; Pascual et al., 2006). In a recent issue of *Molecular Cell*, Carstens and colleagues describe the exciting identification of two new, related cell-type-specific splicing regulators that induce the epithelial splicing pattern of several target mRNAs (Warzecha et al., 2009). This work not only substantially expands the list of tissue-specific splicing regulators to include proteins that are predominantly epithelial, but in so doing, sets the stage for a deeper look into the regulation of cell-type-specific alternative splicing and the physiologic consequences of such genetic control.

One of the best documented examples of tissue-specific alternative splicing is the mutually exclusive inclusion of exons IIIb and IIIc of *FGFR2* (fibroblast growth factor receptor 2), which are expressed in either epithelial (IIIb) or mesenchymal (IIIc) tissues and recognize distinct ligands (Orr-Urtreger et al., 1993). Work from several groups has identified many ubiquitous splicing factors that influence the choice of *FGFR2* exon inclusion (Figure 1; Hovhannisyan and Carstens, 2007; Mauger et al., 2008 and references therein). However, none of these proteins fully explains the tight tissue-specific

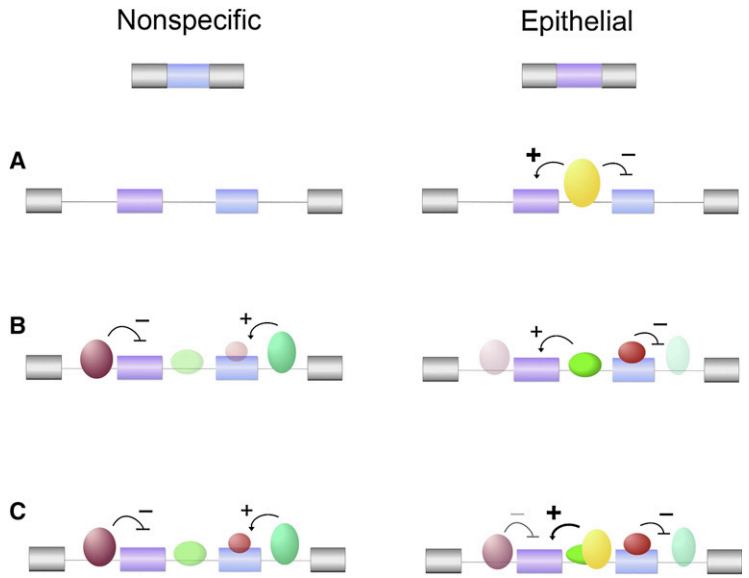

Figure 1. Potential Mechanisms for Achieving Tissue-Specific Splicing Patterns
Differential splicing of two exons in an epithelial versus nonepithelial (nonspecific) pattern could theoretically be achieved by (A) an epithelial-specific primary regulator that drives inclusion of an otherwise unused exon and represses use of a "default" exon, (B) tissue-specific differences in the abundance and balance of multiple ubiquitous regulatory factors, or (C) a tissue-specific regulatory protein (yellow) that augments or counters the activity of other ubiquitous factors. The regulation of FGFR2 is most consistent with (C), in which the yellow protein corresponds to ESRP1/2, and other regulatory proteins include PTB, hnRNP A1, Fox, Tia-1, hnRNP M, and hnRNP F/H (see Warzecha et al. [2009] and references therein).

control of *FGFR2* splicing. To seek additional players in the epithelial-specific splicing of *FGFR2*, Warzecha et al. utilized a high-throughput cDNA expression screen in a cell line harboring a reporter construct that allowed luciferase expression only in the case of exon IIIb inclusion. Strikingly, this screen identified 18 unique cDNAs that were sufficient to induce expression of exon IIIb in a cell line that normally expressed only exon IIIc. Of these positive clones, two related genes, *Rbm35a* and *Rbm35b* (later termed epithelial splicing regulatory proteins 1 and 2 [ESRP1/2]) were chosen for further study based on the fact that their induction of IIIb inclusion was dependent on the presence of a known exon IIIb enhancer element within the RNA. Both ESRP1 and -2 were able to bind this RNA sequence, but not a mutated version, providing evidence for direct regulation.

Remarkably, ESRP1/2 appear to be epithelial-specific proteins (hence the renaming), as both qPCR and in situ hybridization revealed a high level of *ESRP1/2* mRNA expression only in epithelial tissues and cell lines. Furthermore, expression of *ESRP1/2* mRNA decreased in a model of epithelial-to-mesenchymal transition (EMT). Unfortunately, antibodies to cleanly detect endogenous ESRP1/2 proteins are currently not available; therefore, rigorous proof of cell-type-specific expression of the protein products awaits further studies. However, consistent with tissue-specific activity of ESRP1/2, knockdown of ESRP1/2 in epithelial cells led to a switch in splicing to the mesenchymal *FGFR2* isoform (exon IIIc), and conversely, overexpression of ESRP1/2 in cells lacking their endogenous expression induced the *FGFR2* epithelial splicing pattern (exon IIIb).

Importantly, knockdown of ESRP1/2 in epithelial cell lines also induced the nonepithelial pattern of splicing of three additional pre-mRNAs tested. Furthermore, these same three genes showed a change in isoform expression when epithelial cells were induced to transition to mesenchymal cells, and this change was reversed upon ectopic expression of ESRP1/2. Together, these data suggest that ESRP1/2 are regulators of concerted epithelial-specific splicing events, although the extent and nature of such regulation remains unknown. Mapping of the ESRP1/2-binding site within these four known ESRP-regulated RNAs and large-scale analysis of mRNA from cells depleted for ESRP1/2 will be an important next step to assess whether these genes are indeed direct targets of ESRP1/2 and to determine the scope of their effect on epithelial splicing. Interestingly, the ESRP *Drosophila* homolog Fusilli was able to substitute for ESRP1/2 in overexpression studies, indicating a conserved mode of action as well as conserved RNA-binding sites. As Fusilli has also been shown to be highly enriched in epithelial tissues in flies, it is possible that a program of epithelial splicing events has been highly conserved through evolution and may play a more profound physiologic role than previously recognized.

It is worth noting that the initial screen by Warzecha et al. identified over a dozen other proteins that were also able to induce exon IIIb inclusion but were not analyzed further. Indeed, the remarkably high percentage of confirmed positives coming out of their screen (18 of 22 initial hits) suggests a robust assay design that may be applicable in other systems to address similar questions. However, it is interesting to note that none of the previously characterized *FGFR2* regulatory proteins were identified in the screen. This is most likely due to an important caveat of screening for splicing regulators, namely that many are difficult to overexpress or knockdown efficiently due to homeostatic autoregulation and/or loss of cell viability. Importantly, this suggests the screen was not saturating of all *FGFR2* regulatory proteins and that even more may exist.

The presence of further IIIb-inducing proteins is consistent with a combinatorial mode of splicing regulation in which a number of RNA-binding proteins are involved in regulating the outcome of *FGFR2* alternative splicing, with ESRP1/2 anticipated to have a strong, but not absolute, influence on the ultimate decision. Even if many of the additional genes identified in the current screen turn out to be indirect regulators of IIIb inclusion, ESRP1/2 are only two of several proteins that have been shown thus far to directly regulate *FGFR2* splicing (Figure 1). Similarly, the decision of whether to include exon IIIb or IIIc has been shown to be dependent on the sum of many different regulatory elements within the pre-mRNA. Therefore, to truly understand the mechanism by

which multiple protein inputs "code" for the final splicing outcome, further studies will be required to identify the precise binding sites for ESRP1/2, differentiate whether ESRP1/2 are redundant or have distinct activities, determine how the binding or activity of this protein(s) affects neighboring proteins along the RNA, and establish the mechanism by which all of the *FGFR2* regulatory proteins function.

Finally, the role of cell-type-specific splicing events in determining cell identity will be important and challenging to address. One key question is whether the correct splicing pattern of ESRP1/2 target genes is essential for epithelial differentiation and/or maintenance or, rather, is simply a consequence of differentiation and involved in fine-tuning of cellular function. In their current work, Warzecha et al. have not addressed this topic, but by identifying ESRP1/2 as new splicing regulators in epithelial cells, they have laid the groundwork for future studies.

REFERENCES

Hovhannisyan, R.H., and Carstens, R.P. (2007). J. Biol. Chem. *282*, 36265–36274.

Li, Q., Lee, J.A., and Black, D.L. (2007). Nat. Rev. Neurosci. *8*, 819–831.

Matlin, A.J., Clark, F., and Smith, C.W. (2005). Nat. Rev. Mol. Cell Biol. *6*, 386–398.

Mauger, D.M., Lin, C., and Garcia-Blanco, M.A. (2008). Mol. Cell. Biol. *28*, 5403–5419.

Orr-Urtreger, A., Bedford, M.T., Burakova, T., Arman, E., Zimmer, Y., Yayon, A., Givol, D., and Lonai, P. (1993). Dev. Biol. *158*, 475–486.

Pan, Q., Shai, O., Lee, L.J., Frey, B.J., and Blencowe, B.J. (2008). Nat. Genet. *40*, 1413–1415.

Pascual, M., Vicente, M., Monferrer, L., and Artero, R. (2006). Differentiation *74*, 65–80.

Ule, J., Ule, A., Spencer, J., Williams, A., Hu, J.S., Cline, M., Wang, H., Clark, T., Fraser, C., Ruggiu, M., et al. (2005). Nat. Genet. *37*, 844–852.

Wang, E.T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S.F., Schroth, G.P., and Burge, C.B. (2008). Nature *456*, 470–476.

Warzecha, C.C., Sato, T.K., Nabet, B., Hogenesch, J.B., and Carstens, R.P. (2009). Mol. Cell *33*, 591–601.

It Takes Two Binding Sites for Calcineurin and NFAT to Tango

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In a recent issue of *Molecular Cell*, Rodríguez et al. (2009) identified the NFAT LxVP motif binding site as the same composite surface formed by the two calcineurin subunits that is recognized by the cyclophilin-CsA and FKBP-FK506 complexes.

The molecular choreography between different proteins involved in signal transduction comes in many varieties. Thus, when the music is turned on upon the engagement of the T cell receptor with the MHC-antigen complex, a dance party between the various signaling proteins begins within the cytosolic and the nuclear compartments of T lymphocytes. The majority of them prefer "rock and roll"—partners barely touch one another, and when they do, the contact is very brief and transient. However, some seem to enjoy the "cha-cha-cha," holding one hand of their dancing partners. For a long time, the steps preferred by a unique pair of signaling partners, the protein phosphatase calcineurin and its most celebrated substrate, NFAT, remained a mystery. Now, new work from Rodríguez et al. (2009), in conjunction with previous work by others, reveals that calcineurin and NFAT tango while holding two "hands."

Calcineurin is a unique calcium- and calmodulin-dependent protein phosphatase that transmits calcium signals from the cytosol to the nucleus to regulate gene expression in T cells, neurons, and muscle cells. Since its identification as the common target of the widely used immunosuppressive drugs cyclosporin A (CsA) and FK506 (Liu et al., 1991), calcineurin has posed one puzzle after another to biologists and chemists alike concerning its unusual interactions with two structurally unrelated cyclophilin-CsA and FKBP-FK506 complexes and the molecular mechanism by which it catalyzes NFAT dephosphorylation. The crystal structures of the ternary complexes between calcineurin and the two immunophilin-drug complexes revealed that both cyclophilin-CsA and FKBP-FK506 bind the same composite hydrophobic surface formed by an amphipathic peptide extruding from the C terminus of the calcineurin catalytic domain and its regulatory subunit that is itself a calmodulin homolog (Griffith et al., 1995; Huai et al., 2002; Jin and Harrison, 2002; Kissinger et al.,