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An Alternative Splicing Network Links Cell-Cycle Control to Apoptosis

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SUMMARY

Alternative splicing is a vast source of biological regulation and diversity that is misregulated in cancer and other diseases. To investigate global control of alternative splicing in human cells, we analyzed splicing of mRNAs encoding Bcl2 family apoptosis factors in a genome-wide siRNA screen. The screen identified many regulators of *Bcl-x* and *Mcl1* splicing, notably an extensive network of cell-cycle factors linked to aurora kinase A. Drugs or siRNAs that induce mitotic arrest promote proapoptotic splicing of *Bcl-x*, *Mcl1*, and *caspase-9* and alter splicing of other apoptotic transcripts. This response precedes mitotic arrest, indicating coordinated upregulation of prodeath splice variants that promotes apoptosis in arrested cells. These shifts correspond to post-translational turnover of splicing regulator ASF/SF2, which directly binds and regulates these target mRNAs and globally regulates apoptosis. Broadly, our results reveal an alternative splicing network linking cell-cycle control to apoptosis.

INTRODUCTION

Nearly all human precursor messenger RNAs (pre-mRNAs) undergo alternative splicing (AS), with tremendous variation and specificity across tissues, development, and disease (Pan et al., 2008). This vast complexity is a formidable challenge to experimental and informatic analysis of AS and its physiological roles (Blencowe, 2006). One emergent concept is that functionally coherent transcript populations, termed RNA "regulons," are coregulated by dedicated RNA-binding proteins (RBPs) to promote specific biological functions (Keene, 2007). These RBPs, notably SR proteins and hnRNPs, "decode" transcript *cis* elements and control stepwise assembly of spliceosomal snRNPs: U1 at the 5′ splice -site (ss), then U2 near the 3′ ss, and finally U5/4/6 (Wahl et al., 2009; Black, 2003). Splicing control is integrated with signal transduction pathways, promoting dynamic, context-driven regulation. Collectively, these features produce robust cell- and tissue-specific signatures of exon use that shape many aspects of cell fate (Moore and Silver, 2008). Exon signatures are radically transformed in tumors, but the causes and consequences are unknown.

In this study, we examine AS of Bcl2 family apoptosis regulators *Bcl-x* and *Mcl1*. Bcl2-like proteins contain up to four Bcl2-homology (BH) domains (BH1–4) (Hardwick and Youle, 2009). Factors possessing all four BH domains, including Bcl2, Bcl-xL, and Mcl1L, antagonize apoptosis by preventing mitochondrial outer membrane permeablization (MOMP), thus sequestering proapoptotic factors in mitochondria. Factors lacking one or more BH domain, including Bid, BAD, and BAX, are proapoptotic and promote MOMP. A finely tuned balance of pro- and antiapoptotic Bcl2-like factors therefore controls mitochondrial integrity and hence downstream steps in apoptosis such as apoptosome formation and caspase activation (Wang and Youle, 2009). Remarkably, *Bcl-x*, *Mcl1*, and some other *Bcl2* family mRNAs are alternatively spliced to yield both long (L) antiapoptotic and short (S) proapoptotic forms. For *Bcl-x*, use of an alternative 5′ ss in exon 2 excludes the BH1 and BH2 domains (Akgul et al., 2004). For *Mcl1*, exon 2 skipping excludes the BH1 and BH2 domains and eliminates the downstream transmembrane domain via frame shift.

Many *cis*-regulatory elements and *trans*-acting factors exert combinatorial control of *Bcl-x* splicing. Most known regulators, including Sam68, ASF/SF2, hnRNP F/H, SRp30c, and RBM25, altered *Bcl-x* AS in vitro or when overexpressed in cell culture (Cloutier et al., 2008; Zhou et al., 2008; Paronetto et al., 2007; Garneau et al., 2005). In addition, in RNA interference (RNAi)-based loss-of-function assays, depletion of Sam68 and hnRNPA1 favored *Bcl-xL* formation, while depletion of U2 snRNP component SF3B1/SAP155 favored *Bcl-xS* (Paronetto et al., 2007; Massiello et al., 2006). Comparably little is known of *Mcl1* splicing regulation.

Beyond Bcl2-like factors, caspases, "death receptors," ligands and various adaptors are regulated by AS, suggesting broad roles in controlling apoptosis (Schwerk and Schulze-Osthoff, 2005). Many apoptosis regulators, including Bcl2-like proteins, are proto-oncogenes that contribute to apoptosis resistance in cancer (Letai, 2008; Fesik, 2005). Modulation of apoptotic factors by targeting the splicing machinery is thus an attractive strategy to facilitate tumor cell death. Furthermore, while the divergent functions of *Bcl-x* and *Mcl1* isoforms in apoptosis are well established, the physiological contexts and upstream regulation of their expression are poorly defined.

These unanswered questions illustrate a pervasive challenge in defining physiological contexts of AS regulation, because strategies for systematic evaluation of upstream regulation are limited.

Genome-scale screening of RNA regulatory events is complicated by the difficulty of visualizing RNAs in vivo, and the technical infeasibility of high-throughput measurements by RT-PCR and other methods. Splicing-sensitive fluorescent reporters are an alternative strategy that produce a robust, visual output suitable for screening efforts (Stoilov et al., 2008; Warzecha, et al., 2009; Orengo et al., 2006). Here, we present high-throughput assays that recapitulate physiological regulation of *Bcl-x* and *Mcl1* AS. In a whole-genome siRNA screen, we identified new factors that regulate the balance of anti- and proapoptotic splice isoforms, with striking enrichment for cell-cycle factors. These results define functional interactions between the cell cycle and splicing machineries in human cells that manifest in a coordinated program of AS controlling apoptosis.

RESULTS

Reporter Assays for *Bcl-x* and *Mcl1* Alternative Splicing

To develop splicing assays for high-throughput analysis, we designed splicing-sensitive reporters for the Bcl2 family apoptosis regulators *Bcl-x* and *Mcl1*. 5′ untranslated region (UTR), open reading frame (ORF), and intervening intron sequences for *Bcl-x* and *Mcl1* were cloned in C-terminal fusions with *Venus* (yellow) and *mCherry* (red) complementary DNAs (cDNAs), respectively (Figure 1A). In HeLa cells, these constructs expressed long and short spliced mRNAs at ratios similar to endogenous mRNAs (Figure 1B). To render constructs splicing-sensitive, premature termination codons (PTCs) were introduced in alternative exon regions exclusive to long splice forms. As expected, PTCs eliminated expression of long protein variants, but short forms were retained (Figure 1C).

To produce screen assay cell lines, splicing reporters were stably transfected into HeLa cells along with a constitutive mCerulean fluorescent protein (CFP) construct. All constructs used the human EF1α promoter, which contains a 5′ UTR intron, allowing dual measurements of minigene splicing and a constitutively spliced CFP reporter under identical control. To test the *Bcl-x* reporter line, we verified that siRNA-depletion of known regulator SF3B1 increased Bcl-xS-Venus expression relative to a non-targeting control (Figure 1D, left panels) (Massiello et al., 2006). Immunoblotting confirmed efficient siRNA knockdown of SF3B1, and upregulation of Bcl-xS-Venus reporter protein (Figure 1E). RT-PCR confirmed upregulation of the *Bcl-xS-Venus* mRNA (Figure 1F). Finally, RT-PCR analysis of endogenous *Bcl-x* transcript in HeLa cells verified that SF3B1 knockdown shifted splicing toward *Bcl-xS*, demonstrating congruous regulation of minigene and endogenous splicing (Figure 1G, upper panel).

We had no a priori knowledge of *Mcl1* regulators, but SF3B1 knockdown also upregulated Mcl1S-mCherry in the splicing assay, establishing a positive assay control (Figure 1D, right panels). Analysis of endogenous *Mcl1* verified this shift toward *Mcl1S* (Figure 1G, lower panel).

High-Throughput siRNA Screens for Alternative Splicing Regulators

To identify regulators of *Bcl-x* AS, >21,000 siRNA pools targeting known and predicted human genes were screened for upregulation of the *Bcl-x* reporter (Figure 2A). Three hundred and sixty-nine positive hits were identified with a Support Vector Machine (SVM) model that determined reproducibility (i.e., ''confidence'') across triplicates and signal ''strength'' relative to positive and negative control siRNAs (Figure 2B, and Figure S1 and Table S1 available online). Hits had strong gene ontology (GO) enrichments for mRNA splicing/processing, protein kinase signaling, cytoskeleton association, and cell-cycle functions (Figure 2C). Importantly, the screen blindly recovered positive control SF3B1 and several of its interactors.

For validation, hits were retested in the screen assay with four individual siRNAs from deconvoluted SMARTpools. Two hundred and seventy-four of 369 factors validated with at least one siRNA, and 160 validated with two or more (Figure 2A and Table S2). In the primary and validation screens, Bcl-x-Venus expression correlated significantly with cell death (Figures S2A and S2C). Venus showed no systematic correlation to CFP, indicating that nonspecific promoter effects were not a major source of positives (Figures S2B and S2F). In the validation screen, apoptosis was tracked by Annexin-V-Cy5 staining and showed strong coupling to proapoptotic *Bcl-x* splicing and cell death (Figures S2D and S2E). Functional enrichments, notably splicing and cell-cycle regulation, were similar between validated hits and the primary screen (Figure 2C). Subsequent analyses focused on ''high-confidence'' factors validated with two or more siRNAs. We focused specifically on aurora kinase A (AURKA) and other mitotic regulators because of their strong functional enrichment and their novelty in the context of splicing regulation.

Physiological function for screen hits was confirmed by analysis of endogenous *Bcl-x* AS in HeLa cells. siRNAs against 19 factors were tested, and 16 ($\sim$85%) significantly shifted *Bcl-x* splicing toward *Bcl-xS* (Figure S3A). The magnitude of these shifts matched or exceeded fold changes that promote apoptosis in various cell types, indicating physiological significance (Mercatante et al., 2002; Taylor et al., 1999). As further indication of this significance, AURKA knockdown strongly shifted endogenous Bcl-x protein toward Bcl-xS (Figure S3B). This shift was larger than the corresponding mRNA shift, suggesting an amplification effect during translation. Regulation of endogenous *Bcl-x* by AURKA and other hits also confirmed in MCF7 (breast adenocarcinoma) and PANC1 (pancreatic carcinoma) cells (Figure S3C). These lines differ markedly in steady state AURKA levels, with MCF7 (like HeLa) expressing high levels, and PANC1 levels closer to ''normal'' tissue (Ross et al., 2000). Thus, splicing regulation by AURKA inhibition was not restricted to HeLa cells and occurred irrespective of steady state AURKA levels.

Apoptosis Suppression Defines Direct Regulators of *Bcl-x* Alternative Splicing

In screening experiments, proapoptotic *Bcl-x* splicing correlated to apoptosis induction and increased cell death (Figure S2). However, screen hits are likely to include both direct regulators