Genome-wide RNAi screen discovers functional coupling of alternative splicing and cell cycle control to apoptosis regulation

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Alternative splicing (AS) is widely recognized as a major source for biological diversity and regulation. The contribution of AS to eukaryotic development and complexity is likely even greater than previously recognized, as a recent report demonstrates that >90% of human genes are alternatively spliced.¹ The regulation of AS is under the control of cis-elements residing in mRNA sequences and a large repertoire of RNA binding proteins (RNABPs).² AS regulation is further integrated with signal transduction and cell-specific cues that control RNABP dynamics and function.³ While many assays have revealed mechanisms for individual AS regulatory events, global understanding of AS regulation is still at an early phase.³ Specifically, systematic identification of AS regulators, particularly those upstream of RNABPs, has been difficult via hypothesis-driven studies. Therefore, even in some of the best studied examples of AS it is unclear how AS is connected to other biological processes to carry out complex cellular functions.

We recently completed a whole-genome siRNA screen using a novel reporter strategy to identify factors that regulate AS at all levels. As model AS events, we examined mRNAs for *Bcl-x* and *Mcl1*, *Bcl-2* family apoptosis regulators that possess two splice isoforms—anti-apoptotic *Bclx-L* and *Mcl1-L*, and pro-apoptotic *Bclx-S* and *Mcl1-S*.⁴ In the reporters, Venus and mCherry fluorescent protein cDNAs were fused to the 3' end of *Bclx* and *Mcl1* genomic sequences, respectively. Premature termination codons (PTC) were then introduced to the alternatively

spliced regions exclusive in the long isoforms (Fig. 1A). Thus, *Bclx-S* and *Mcl1-S* could be detected as fluorescence fusion proteins, but the PTC prevented expression of fluorescence-tagged long isoforms. A genome-wide siRNA screen using the *Bclx* reporter revealed 160 factors, that when depleted, led to upregulation of pro-apoptotic *Bclx-S* splicing. These factors were further tested for the *Mcl1* reporter, identifying 52 factors that coordinately regulate AS. Surprisingly, knockdown of many cell cycle regulators, including the potent oncogene aurora kinase A (AURKA), dramatically increased *Bclx-S* and *Mcl1-S* isoforms. Further analysis of factors downstream of AURKA linked the splicing changes to direct regulation by the splicing factor ASF/SF2 (SFRS1). Upon AURKA inhibition, ASF/SF2 was degraded, causing a coordinated shift toward pro-apoptotic splicing in *Bclx* and other apoptosis-related splicing events.

Interestingly, pharmacological inhibition of AURKA by VX-680 (aurora kinase inhibitor) showed that accumulation of pro-apoptotic *Bclx-S* splicing was concomitant to mitotic arrest. However, in actively cycling G₂/M cells, *Bclx-S*-Venus showed no increase. Also, *Bclx-S* upregulation was not detected during a double-thymidine block that caused S-phase arrest, indicating the specificity of the splicing response to mitotic arrest. Since the current view holds that splicing and transcriptional activity are mostly suppressed in mitosis,⁵ we went on to ask if the splicing response preceded mitotic arrest, or whether it could represent a downstream effect of aberrant division in cells that were not yet

arrested. Cells were synchronized with double-thymidine, released and treated with VX-680; *Bclx-S* upregulation was found to be comparable to asynchronous cells. Here, the venus-fluorescence was measured 16 hours post thymidine-release, preventing one intervening cell cycle. These data excluded the possibility that the splicing shift was caused by a subset of cells undergoing abnormal cell division before mitotic arrest. Thus, it can be concluded that the *Bclx-S* splicing response preceded mitotic arrest. Consequently, our investigation suggests that pro-death factors accumulate prior to mitotic arrest. This finding provides novel mechanistic clues for how apoptosis occurs in arrested cells, and it is likely that this mechanism extends to other apoptosis regulators.

Aberrant AS is implicated in cancer,⁶ but its specific functions are not well understood. In this screen, we found numerous factors that are themselves oncogenes or are frequently misregulated in cancer. Specifically, protein interaction analysis on the top hits showed an AURKA-centered network spanning cell cycle regulators, spliceosome components and tumor suppressors. While aberrant regulation of AURKA is well known to induce spindle checkpoint abrogation, spindle assembly defects and chromosome instability in tumors,⁷ our data indicate that AURKA can also contribute to oncogenesis by promoting anti-apoptotic splicing of crucial apoptosis regulators in malignant cells. Conversely, inhibition of AURKA leads to a dramatic elevation in pro-apoptotic splicing isoforms followed by mitotic arrest. Since AURKA is

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Figure 1. (A) Splicing reporters *Bcl-x* and *Mcl1* are schematized. Green rectangles are exons, with gray areas depicting alternative spliced region. Black lines between rectangles are introns. Arrows denote translational start site. Inserted PTCs exclusive to long isoforms are shown by red "stop signs." (B) A model of the functional coupling of cell cycle control and AS to apoptosis regulation from our genome-wide screen is shown.

a well-known target for anti-tumor therapies, our findings may be useful for new insights in drug development and therapeutic targeting of AURKA.

Taken together, our screen revealed a novel physiological association between the cell cycle and AS. Interestingly, previous studies have hinted at a possible reciprocal link between cell cycle control and AS regulation. For example, many cell cycle regulators have been identified as spliceosome components, including CDC40 and CDC5L, which were strong hits in our study. Moreover, recent large-scale screens identified splicing factors as affecting cell cycle progression and mitotic spindle integrity.^{8,9} However, a

functional connection between cell cycle and AS has not been emphasized in the literature. Our genome-scale exploration of apoptosis-related AS unexpectedly identified many cell cycle regulators, whose functions spanned multiple aspects of cell cycle control, including regulators of the G₂/M transition, mitotic spindle assembly and microtubule transport. Importantly, downstream of the cell cycle regulators, we discovered an ASF/SF2 mediated 'RNA regulon'* consisting of coordinated *Bclx*, *Mcl1*, *caspase-9* and other apoptosis regulator RNAs. Through this 'RNA regulon', cells respond to upstream signals of cell cycle disruption and carry out a pro-apoptotic AS splicing shift in the RNA

members, inducing apoptosis (Fig. 1B). Broadly, our screen revealed a novel functional coupling of cell cycle control and AS, where these highly regulated processes are coordinated to regulate apoptosis.

*RNA regulon: 'RNA regulon' hypothesis proposes that groups of functionally related mRNAs are coordinated and regulated by specific, shared RNABPs to quickly react to environmental cues.¹⁰

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