Chapter I. Introduction

1.1. Post-transcriptional processing plays a significant role in regulating gene expression

Proper control of gene expression is essential for normal cellular function, development and reproduction. Eukaryotic gene expression is regulated at both the transcriptional and post-transcriptional level. Post-transcriptional regulation arises through cellular control of RNA processing, splicing, export, localization, and turn-over[1], and allows cells greater control over gene expression patterns[2]. mRNA splicing, export, and turn-over are tightly coupled processes. Alternative splicing creates proteomic diversity and controls protein isoform levels by regulating the patterns in which exons are assembled. Cells can also control gene expression by varying mRNA turnover rates for specific transcripts through nonsense-mediated decay (NMD), a cellular surveillance system in which mRNAs containing premature termination codons are selectively degraded. Recent studies indicate that alternative splicing and NMD are often coupled such that gene expression can be controlled in both a spatial and temporal manner[3].

1.2. Pre-mRNA splicing

Mature eukaryotic mRNAs are formed from precursor mRNAs (pre-mRNAs) that contain introns and exons. The intron sequences are removed and the exon sequences are ligated together to form a mature mRNA in a process called splicing (Figure 1.1a). A typical human gene contains relatively short exons (50–250 base pairs (bp)) which are

separated by significantly longer introns (hundreds to thousands of bp)[4]. Splicing requires exon recognition followed by accurate cleavage at exon-intron boundaries which are determined by the nearly invariant GU and AG intronic dinucleotides at the 5' and 3' exon-intron junctions, the polypyrimidine tract $(Y)_n$ and the A residue (adenosine) that serves as the branch point[5,6]. Trans-acting factors are recruited to assemble across splice sites, forming a catalytically active complex known as the spliceosome which is responsible for the excision of introns and recombination of exons[7]. The spliceosome is composed of five small nuclear ribonucleoprotein particles (snRNPs) and over 100 protein factors[8,9] and utilizes RNA–RNA, RNA–protein, and protein–protein interactions to correctly excise introns and to splice exons[10].

The components of the spliceosome coordinate the stepwise associations, dissociations and conformational changes of the pre-mRNA, snRNPs, and protein complexes necessary for splicing and transcript release to occur[9]. As seen in Figure 1.1b, intron removal takes place in two S_N2-type transesterification reactions. In the first step, the 5' splice site is attacked by the 2'-hydroxyl of the branch site adenosine, generating the exon 1 intermediate and a branched intron 'lariat' attached to exon 2. In the second step, the 3' splice site is attacked by the 3'-hydroxyl of the free exon 1 intermediate. The final products are the spliced mRNA and the excised intron in lariat form [11].

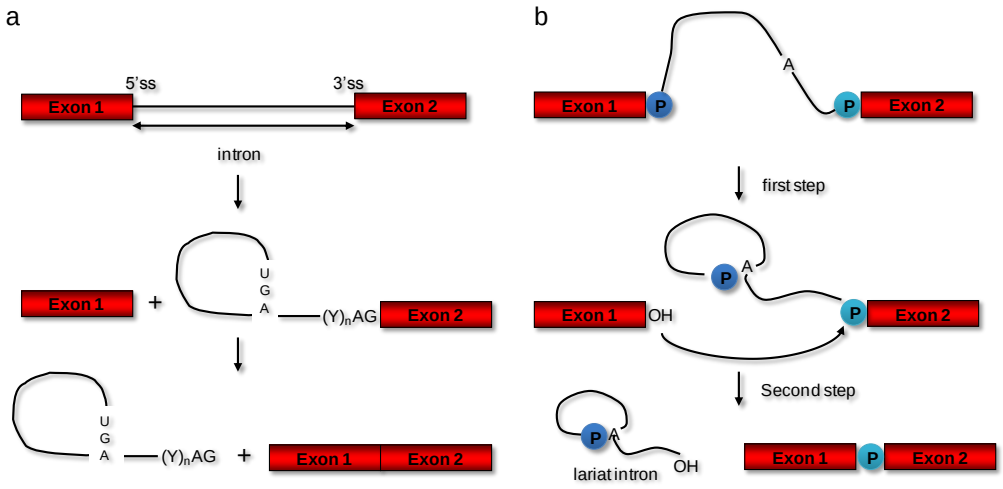

Figure 1.1. Mechanism of splicing. (a) Consensus mammalian 5' splice site (5' ss), branch point (A), polypyrimidine tract (Y_n), and 3' splice site (3' ss) sequences are shown. Splicing takes place in two transesterification steps. The first step results in two reaction intermediates: the detached 5' exon and an intron 3'– exon fragment in a lariat structure. The second step ligates the two reactions and releases the intron lariat. (b) Two-step transesterification pathway of pre-mRNA splicing. First, the phosphodiester bond at the 5' ss is attacked by the 2'-hydroxyl of an adenosine at the branch point, which generates a free 5' exon and an intron lariat-3' exon. Subsequently, the 3'-hydroxyl of the 5' exon attacks the phosphodiester bond at the 3' ss, leading to exon ligation and an intron lariat-3' exon.

1.3. Basic principles of alternative splicing

Alternative splicing is a process by which multiple protein isoforms are generated from a single coding region by altering the ways in which the exons are joined together,

or defined, during the splicing process[12]. Alternative splicing allows for the enrichment of the transcriptome and proteome of higher organisms without the need for genome expansion[9]. The potential of alternative splicing to generate more protein isoforms from a single gene than the number of genes in an entire organism helps to explain the discrepancy between the low number of human protein-coding genes (~26,000) and the number of human proteins, which is estimated to be more than 90,000[13,14]. Recent studies using high-throughput deep sequencing suggest that the extent of alternative splicing is significantly greater in humans than was previously estimated with approximately 92–94% of human multi-exon genes being alternatively spliced[15,16]. While these studies have provided catalogues of splicing events, further characterization is needed to determine if these spliced isoforms have a particular function[17,18]. A current challenge in the field of splicing is the development of high-throughput cell-based assays to evaluate the function of these spliced variants[17].

Alternative splicing occurs through various modes including exon skipping/inclusion, alternative 3' ss, alternative 5' ss, mutually exclusive exons, intron retention, and alternative initial/terminal exons (Figure 1.2)[5,11,19]. Consensus motifs contain only about half of the information required for accurate recognition of exons and introns in human transcripts[20]. It has recently been discovered that human introns contain many sequences that resemble consensus splice site sequences, or 'decoy' splice sites, that are rarely recognized by the splicing machinery[21]. At the level of exon definition, pseudoexons, which are composed of these decoy signals, outnumber real exons by an order of magnitude[22,23]. Despite the potential for errors, the splicing process occurs with high fidelity, implying that there are additional sequences besides canonical elements that

play a role in the splicing of most transcripts. These elements include specific *cis*-acting elements in exons and introns, which aid in splice site recognition and act as enhancers or repressors of splicing. Within the spliceosome, splicing is carried out by the interplay of these *cis*-acting sequences and trans-acting factors that modulate them, leading to a "splicing code"[24].

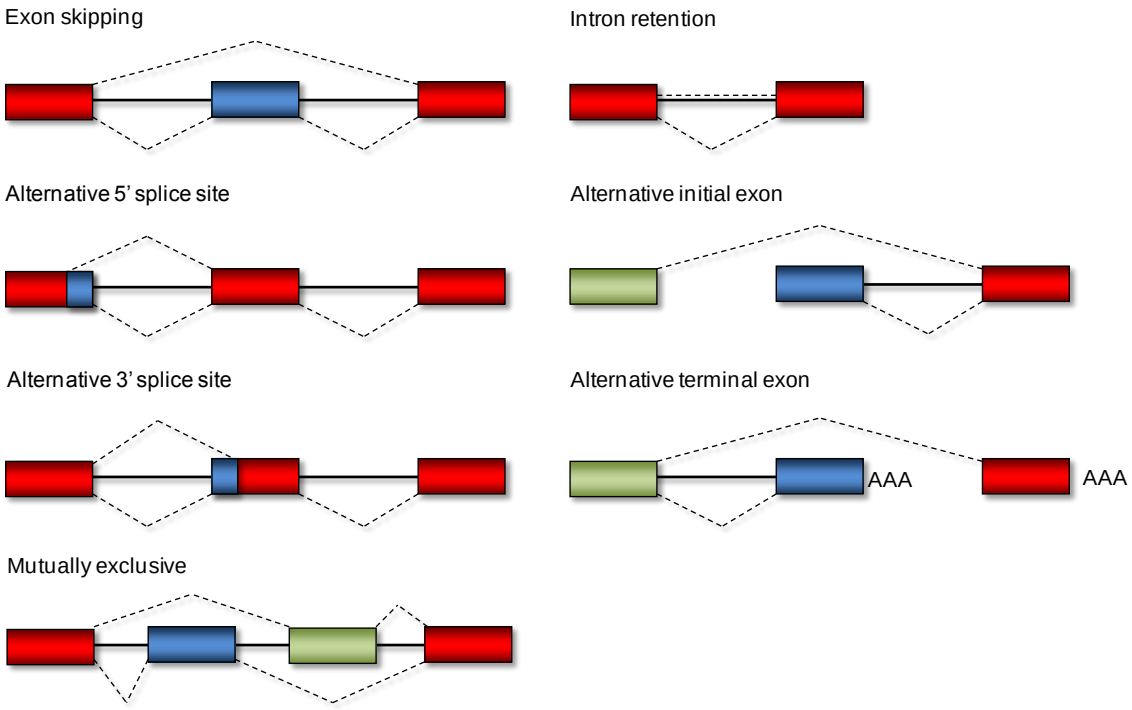

Figure 1.2. Major forms of alternative splicing. These modes are responsible for the generation of functionally distinct transcripts. Labels: introns are represented by solid lines and dashed lines indicate splicing activities.

1.4. Elements of a "splicing code"

The auxiliary elements that make up the splicing code include exonic and intronic splicing enhancers (ESEs and ISEs, respectively), which aid in exon recognition, and exonic and intronic splicing silencers (ESSs and ISSs, respectively), which suppress exon inclusion (Figure 1.3)[11,19]. These splicing regulatory elements (SREs) generally function by recruiting trans-acting splicing factors that activate or suppress splice site recognition or spliceosome assembly[19]. Mutating, removing, and/or shifting the location of these sequences will affect the overall splicing pattern of a transcript[25]. In contrast to the canonical splice sites whose sequence and position are well characterized, the complex code formed by these auxiliary SREs is only partially understood. In addition, the regulatory proteins that interact with these specific sequences to either stimulate or repress exon recognition have been only partially elucidated. Therefore the generation of a complete "splicing code" will require the elucidation of all types of SREs and the corresponding trans-acting factors that regulate them.

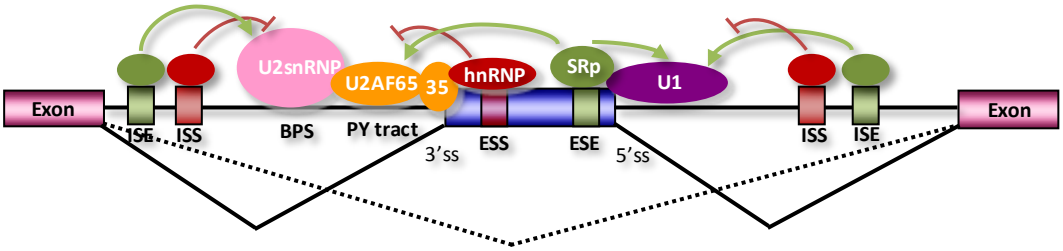

Figure 1.3. Elements of a "splicing code". The diagram illustrates regulated splicing. Green arrows illustrate the positive activity of splicing enhancers (green bars) on the selection of adjacent splice sites in the alternative exon (blue bar). Red arrows with flat bars indicate the negative activity of splicing silencers (red bars) on the regulation of

adjacent splice sites. Splicing is regulated by *cis*-elements (ESEs, ESSs, ISSs, and ISEs) and the trans-acting factors that bind them (SR proteins, hnRNP, and unknown factors).

The most well characterized family of regulatory proteins that bind both intronic and exonic *cis*-elements are the serine/arginine-rich (SR) and SR-like proteins. The human SR family contains ten identified members[26] that are thought to mediate interactions between splicing factors bound to the 5' and 3' splice sites. All SR proteins have a modular structure and contain either one or two copies of an RNA-recognition motif (RRM) and a C-terminal end highly enriched in arginine and serine dipeptides (RS domain)[27]. The RRMs serve to mediate sequence-specific binding to the RNA, which determines substrate specificity, and the RS domains are involved in protein-protein interactions that are essential for the recruitment of the splicing machinery[5]. Most exonic splicing enhancers (ESEs) are purine rich and different ESEs are recognized by various subsets of SR proteins[8]. Additionally, SR protein functions have been extended to mRNA export[28], mRNA stability[29], protein translation[30], and nuclear export[31].

The heterogeneous nuclear ribonucleoproteins (hnRNPs) are a diverse class of proteins that bind to both exonic splicing silencers (ESSs) and intronic splicing silencers (ISSs) [12]. Similar to SR proteins, hnRNPs have a modular structure consisting of one or more RRMs that may be involved with RNA binding and domains that are presumed to mediate interactions with other proteins. Amongst the best characterized members of this family are hnRNP A1[32] and PTB (hnRNP I)[33,34]. This family of proteins function by a variety of mechanisms and often serve as antagonists to SR proteins through competitive

binding to the transcript[35]. These factors can often block essential interactions between spliceosome components to inhibit splicing[36,37].

Overexpression of SR proteins and hnRNP proteins has been shown to affect the splicing patterns of alternatively spliced pre-mRNAs *in vivo*[38]. Most hnRNP and SR proteins shuttle continuously between the nucleus and cytoplasm and consequently their subcellular distribution can shift in response to stress signals[9]. Recent studies have also demonstrated that the relative amounts of SR and hnRNP A/B proteins are important in regulating patterns of alternative splicing in a tissue-specific and developmentally-regulated manner[39] . The expression of these proteins is unique to each cell type and thus the regulation of expression and activity of these proteins is critical for normal alternative splicing and cellular function. Moreover, a diverse set of diseases are associated with changes in expression of trans-acting splicing factors[40]. Disease-related changes in splicing factors are potentially useful biomarkers for disease diagnosis and classification. It has been suggested that the modulation of the relative stoichiometries of splicing factors can be used to regulate the alternative splicing of disease-relevant mRNAs[9]. As such, high-throughput small molecule screens are currently being developed to select for agents that modulate splicing factor ratios towards targeted therapies[41].

1.5. Nonsense-mediated decay is a surveillance pathway in eukaryotes

Studies have indicated that approximately a third of alternative mRNA isoforms are targets for NMD, an mRNA surveillance system that targets aberrant transcripts[3,42].

The NMD pathway targets aberrant transcripts and removes potentially harmful truncated versions of proteins[43]. A recent investigation of the human genome sequence and databases of expressed sequence tags (ESTs) identified a coupling between NMD and alternative splicing[3]. NMD substrates from aberrant splicing processes include transcripts with retained introns, skipped exons and extended 5' or 3' UTRs[44,45]. NMD specifically targets transcripts containing premature termination codons (PTCs) that can be introduced into transcripts as a result of DNA rearrangements, frameshifts, nonsense mutations or errors during transcription and splicing[45,46]. In higher eukaryotes, PTCs are recognized when they occur 50–55 nucleotides (nt) upstream of an exon-exon junction (Figure 1.4)[47]. The current model of the NMD mechanism is that during pre-mRNA processing the spliceosome deposits exon junction complexes (EJCs) at sites of intron removal. During the first round of translation the ribosome displaces the EJCs in its path and then dissociates from the mRNA at the stop codon[45]. However, if a PTC is present the ribosome will stop and will fail to remove any EJCs downstream from the PTC. Interactions between EJC proteins and several release factors[48] trigger mRNA decay through a deadenylation-dependent pathway[49].

NMD plays a significant role in human diseases and inherited cancers[9]. It has been suggested that 25% of all mutations causing genetic disorders and cancers target corresponding mRNAs to NMD[50]. The ability of nonsense transcripts to be targeted or to evade NMD has an effect on the genotype-phenotype results of these mutations[43,51]. Gain-of-function mutations can occur when nonsense transcripts evade NMD and result in the production of truncated proteins that are potentially harmful to cells. Whereas, loss-of function mutations creating PTCs targeted to NMD can modify the severity of the

disease phenotypes. Therefore, novel therapeutic approaches targeting NMD may be able to modify diseases with similar genetic phenotypes[6]. Therapeutic strategies targeting the coupling of NMD with alternative splicing may be also used to fine tune levels of specific trans-factors including splicing factors and other RNA binding proteins[24].

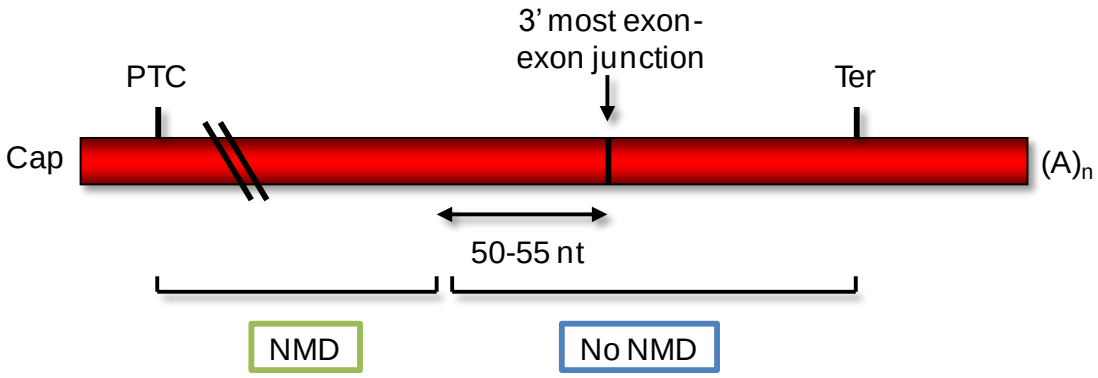

Figure 1.4. NMD and the position of the exon-exon junction. Only the 3'-most exon-exon junction within a generic mammalian mRNA is shown. A PTC that is located in the region indicated in green, which is followed by an exon-exon junction more than 50–55 nt downstream, elicits NMD, whereas a PTC that is located in the region indicated in blue fails to elicit NMD. The normal termination codon (Ter) usually resides in the 3'-most exon.

1.6. Alternative splicing and human disease

The inaccurate recognition of exon-intron boundaries or the failure to remove an intron produces aberrant mRNAs that are either degraded or encode for defective protein isoforms. Studies have suggested that approximately 50% of disease-causing mutations

are found to affect splicing[43]. Mutations affect splicing by disrupting canonical splice sites and auxiliary elements, by creating cryptic splice sites or by altering RNA secondary structure[52]. Mutations that alter trans-acting factors may result in global splicing defects which can have very specific phenotypic outputs[53]. Therefore, understanding the potential effects of single-nucleotide mutations that alter pre-mRNA splicing will enable researchers to develop new therapeutics and treatments that target certain genetic diseases and a variety of cancers[46,54]. Moreover, further elucidation of the *cis*-acting elements that regulate alternative splicing is needed to determine the extent of which single nucleotide polymorphisms (SNPs) modulate splicing through these elements.

1.7. Trans- and *cis*-acting technologies that alter pre-mRNA splicing

Several therapeutic approaches have been developed to alter the splicing patterns of target genes or specific splice variants. Antisense oligonucleotides (AOs) have been used extensively to target alternative spliced transcripts in order to correct disease causing splicing defects. AOs are designed to hybridize and block one or more sequences in the target pre-mRNA that are essential to the splicing event. Several systems based on this general strategy have been used to alter the splice site selection of transcripts involved in disease including breast cancer 1 (*BRCA1*), *SMN*, *β-globin*, the *CFTR* gene in cystic fibrosis, and apoptosis regulator (Bcl-x)[9,29,55]. An antisense technology was recently developed that mimics the functions of SR proteins to restore wild-type splicing in *BRCA1* and *SMN2* pre-mRNA transcripts[46]. This approach, termed ESSENCE (Exon-Specific Splicing ENhancement by small Chimeric Effectors), utilized a peptide-nucleic

acid (PNA) hybrid containing RS dipeptide repeats coupled with an antisense oligonucleotide targeting a mutated ESE in the *SMN2* gene to correct splice site selection [56]. ESSENCE was also demonstrated on the *BRCA1* gene and was successful at suppressing the effects of a mutation on exon skipping *in vitro*.

SMN2 pre-mRNA transcripts have also been targeted by another AO approach which uses a tailed bi-functional antisense oligonucleotide[57]. In this approach, the antisense portion of the oligonucleotide targets the molecule to exon 7 in *SMN2* and the tailed portion contains an ESE element such that splicing factors will be recruited to influence splice site selection. The latter technology was tested successfully *in vitro* and *in vivo*[57,58]. RNAi approaches may also be used to eliminate aberrantly spliced mRNAs by targeting specific isoforms[43]. However, the applications of these and other antisense technologies are limited because of off-target effects, toxicity, efficiency, and issues with delivery.

Alternative splicing is a viable target for pharmacological modulation with small molecules[9]. High-throughput screening strategies have been developed and employed to select for small molecule inhibitors of SR proteins[59], SR protein kinases (SRPKs)[60] and Cdc2-like kinases (CLKs)[61]. These compounds were shown to modulate splicing, although for only a few splicing events. Further large-scale screens are needed to find more potent and specific modulators of alternative splicing which may be used as a general therapeutic approach for treating diseases[9]. In addition, small molecule based therapies have had success as pharmacological agents because they circumvent some of the major issues of delivery that has been encountered by nucleic acids[9]. While recent

studies have employed cell-based selection strategies, these screens were employed on a modest scale and have only examined the splicing patterns of a limited set of genes. The potential of small molecule based strategies targeting alternative splicing can be determined with the development of more robust, quantitative and specific cell-based splicing assays[9].

In addition to trans-acting technologies, *cis*-acting RNA-based regulatory systems have been developed towards the regulation of pre-mRNA splicing in yeast and mammalian cells[62]. Regulation of splicing in these systems is exerted by small molecule responsive aptamers which were inserted into the intronic regions of spliced transcripts. The aptamer sensing components used in these platforms were shown to not be modular and consequently these systems have not been extended to sense other biomolecules. The adaptation of these platforms towards therapeutic applications have also been hampered by the lack of existing aptamers with suitable pharmacological properties, such as limited cell toxicity[62]. The extension of these designs towards applications in health and medicine may be realized with the identification of new aptamers and with the modification of these platforms to be responsive to protein biomarkers.

1.8. *Cis*-acting regulators of alternative splicing

Much effort has been directed toward the characterization of *cis*-acting exonic regulatory sequences. Specifically, *in vitro* and *in vivo* strategies have been implemented to screen for ESEs and ESSs from small randomized libraries[63–67] and within genome

sequence data[68–70]. Identified ESSs are able to control the selection of alternative 5' and 3' ss when placed between competing sites[71]. Results from these *in vivo* studies demonstrate that not all RNA sequences that have been selected against SR proteins are splicing enhancers, underlining the importance of functional screens. Some of the selected exonic splicing regulators displayed significant similarities to naturally occurring regulators, whereas others were novel. From these and other results, it has been suggested that most exons are likely to have multiple regulators (ESEs and ESSs) that act as weak splicing signals which have an additive effect on splicing[26,64]. ESEs and ESSs also play critical roles in directing splicing to consensus splice sites rather than decoy sites[72-74]. However, few exonic regulators may be strong enough on their own to regulate splicing individually[65].

In contrast, fewer ISEs and ISSs have been characterized and little is known regarding the auxiliary factors by which they are bound. These elements are generally short, variable in sequence, individually weak and present in multiple copies[38]. Several common intronic regulator motifs have been observed, including the GGG triplet[75,76], purine-rich motifs[10] or polypyrimidine tracts present in the 3' intronic regions[10]; however, most newly found auxiliary elements tend to be quite degenerate[77]. ISSs and ISEs have been identified near alternatively spliced exons and their mechanistic actions appear to be antagonistic[78]. ISSs may inhibit exon inclusion by recruiting splicing repressors, which directly antagonize splicing factor binding, or by recruiting repressors to multiple binding sites resulting in a 'zone of silencing'[19]. Identified ISSs are variable in sequence and recruit members of the serine/arginine-rich (SR) or the heterogeneous nuclear ribonucleoprotein (hnRNP) protein families[11]. Similar to exonic regulatory elements,

ISSs have been shown to inhibit the inclusion of pseudoexons into mature mRNAs[79]. Several ISEs have been characterized[80], however the nuclear factors that regulate alternative splicing through these sequences have not been elucidated[81]. Further adding to these complexities, are observations that some intronic splicing regulatory elements (ISREs) act as inhibitors upstream of a specific splice site and as enhancers downstream of that splice site[81]. These results support a role for intronic elements in regulating splicing patterns in a combinatorial manner [8]. Despite the widespread importance of ISREs, a systematic experimental characterization or iterative functional screen has yet to be applied towards developing a functional definition of these elements.

1.9. Engineering *cis*-acting intronic regulators of alternative splicing

Cis-acting regulators of alternative splicing play key roles in regulating the form and function of protein isoforms produced from a given gene in response to various signals received by the cell. Therefore, the ability to program alternative splicing patterns will provide a powerful tool to interrogate and manipulate cellular function. Despite the critical role of alternative splicing in creating phenotypic complexity and regulating gene expression, the sequence composition and function of ISREs have not been well elucidated. As a result of this lack of knowledge around how alternative splicing events are encoded at a genetic level, researchers very rarely incorporate control of alternative splicing events in synthetic genetic networks.

My thesis work has centered around the development of strategies for generating synthetic regulators of alternative splicing and for implementing genetic circuits based on the control of alternative splicing events. Chapter II describes the development of a high-

throughput *in vivo* screen for ISRE function. This high-throughput approach combined a systematic screening strategy with extensive genome-wide bioinformatic analyses and experimental characterization. The implementation of this strategy yielded insight into the sequence composition of ISREs, the splicing regulatory networks (SRNs) associated with these sequences and the mechanisms in which they achieve regulation. Chapter III describes an extension of this platform to support the construction of protein-responsive alternative splicing regulatory elements based on the integration of protein-binding RNA aptamers into key intronic locations of a target alternatively spliced transcript. This protein-responsive platform was adapted to detect disease biomarkers, reprogram natural signaling pathways, and control biologically-relevant processes, such as apoptosis, in response to increased signaling through pathways associated with disease. The molecular platforms described in Chapters 2 and 3 represent powerful tools to regulate alternative splicing events and thus gene expression. In addition, the ability to reprogram biological function in response to endogenous protein levels has broad applications in health and medicine, where such molecular tools can provide the basis for the design of targeted "intelligent" therapeutics. Chapter 4 provides a perspective on the general applications of such genetically encoded technologies and future work needed to further characterize these synthetic regulatory systems.

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