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Accurate Splicing of HDAC6 Requires Son

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Accurate splicing of HDAC6 requires Son

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

Vishnu Priya Chowdary Battini

B.E., Vinayaka Mission University, 2010

2013

Wright State University

Wright State University
GRADUATE SCHOOL

November 22, 2013

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY
Vishnu Priya Chowdary Battini ENTITLED Accurate Splicing of HDAC6 requires Son BE
ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
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ABSTRACT

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Accurate Splicing of HDAC6 Requires Son.

Pre-mRNA splicing requires proper splice site selection mediated by many factors including snRNPs and serine-arginine rich (SR) splicing factors. Son is the largest known SR splicing factor, and it has several putative functional domains including an RS domain, a glycine rich patch (G-patch) and double stranded RNA binding domain (DSRBD). One-third of Son's amino acid sequence consists of novel repetitive sequence motifs of unknown function (Sharma et al., 2010). Son is essential for organization of pre-mRNA processing factors in nuclear speckles and for cell cycle progression (Sharma et al., 2010; Sharma et al., 2011; Ahn et al 2011). Exon array analysis of Son-depleted HeLa cells revealed changes in 1061 transcripts showing exon inclusion or exclusion, and a total of 2067 splicing events that are potentially regulated by Son. We validated that Son is required for appropriate splice site choice in transcripts for several chromatin-modifying enzymes, including HDAC6, ADA and SETD8 (Sharma et al., 2011). However, the mechanism by which Son maintains accurate splicing is unknown. We are systematically generating model minigene cassettes for molecular and in situ analysis of Son-dependent splicing regulation. We have constructed a HDAC6 minigene reporter that contains the genomic sequence spanning exons 26 through 29. Following Son depletion in HeLa cells transiently transfected with the HDAC6 minigene reporter construct, we observed skipping of exons 27 and 28 on both the reporter and endogenous HDAC6 transcripts. Stable cell lines constructed using HDAC6 minigene reporter construct showed exclusion of exons 27 and 28 upon Son depletion. In order to study Son-dependent splicing on HDAC6 *in situ*, we aimed to localize splicing factor recruitment to the

HDAC6 reporter minigene transcription site; however, RNA-FISH performed using probes designed to bind to HDAC6 minigene transcripts did not show reproducible labeling of the transcription locus. Finally, HEK293 cells stably expressing four different siRNA-refractory Son deletion mutants were used to show rescue splicing of HDAC6 minigene transcripts.

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CHAPTER 1: INTRODUCTION

Expression of protein coding genes in mammalian cells is a multistep process that begins with transcription of genes into pre-messenger RNAs (pre-mRNAs). Pre-mRNA processing is coordinated with transcription as well as within overall nuclear structure to improve the efficiency of gene expression. Pre-mRNA processing factors modify nascent pre-mRNA to produce a mature messenger RNA (mRNA) that is recognized by the nuclear export machinery and transported to the cytoplasm for translation into protein. Three major steps of pre-mRNA processing include 5' capping, pre-mRNA splicing and polyadenylation at 3' end of mRNA. My project aims to understand how pre-mRNA splicing of a subset of transcripts is regulated by a splicing factor called Son.

Pre-mRNA splicing is the removal of introns from nascent pre-mRNA by a multicomponent-complex called the spliceosome (Reed, 2000; Görnemann et al., 2005). Spliceosomes are comprised of proteins and small nuclear RNAs that are involved recognition of cis-acting elements in pre-mRNAs for intron excision (Wahl et al., 2009). The three important sites within introns that are recognized by components of the spliceosome include the 5' splice site (also called a donor site with consensus 'GU' residues), the 3' splice site (also called an acceptor site with consensus 'AG' residues) and the polypyrimidine tract containing a branch point adenosine sequence. Pre-mRNA splicing begins with recognition of 5' and 3' splice sites in the nascent mRNA by sRNPs, aided by serine-arginine-rich proteins (SR proteins) that help in recruitment of snRNPs to nearby splice sites. The first spliceosome complex formed is the E complex (also called the "commitment complex") containing U1 snRNP that binds to the 5' splice site.

Subsequently, BBP (branch point binding protein), Prp40 and U2AF recognize the branch point sequence, polypyrimidine tract and 3' splice site, respectively. This in turn recruits U2 snRNP, which binds to the branch point sequence forming complex A. Subsequently, recruitment of U4/U6 and U5 snRNPs to complex A forms complex B. Eventually the 5' and 3' splice sites are brought together, followed by the replacement of U1 snRNP by U6 snRNP and the release of U4 snRNP from the complex after which it is called as complex C. Complex C performs the two catalytic reactions of splicing. In the first step, the 5' splice site is cleaved through a trans-esterification reaction and is ligated to an adenosine residue in the branch point sequence to form an intron lariat. In the second step, the 3' splice site is cleaved by another trans-esterification reaction in which the 3' end of the upstream exon is ligated to the 5' end of the downstream exon. The intron lariat is released and degraded via the nonsense mediated RNA decay (NMD) pathway (Reed 2000; Jurica and Moore, 2003; Black 2003).

Alternative splicing is a natural phenomenon in eukaryotes that results in biodiversity of proteins by selective inclusion or exclusion of particular exons or introns during pre-mRNA splicing. Variation in 5' and 3' splice site selection in eukaryotic pre-mRNAs regulates gene expression by altering the coding sequence of mRNA (as reviewed in Black, 2003). The resulting proteins translated from alternatively spliced mRNAs have differential amino acid sequence and often have diverse biological functions. While alternative splicing is influenced by cell type, developmental stage and gender, the machinery used for alternative splicing is not different from the basal splicing machinery. SR proteins and splicing repressors (heterogenous nuclear ribonucleoproteins, or hnRNPs) determine which 5' and 3' splice sites will be targeted by the splicing machinery during alternative splicing. The splice sites in nascent pre-mRNAs are surrounded by several *cis*-acting elements called Exon/Intron Splicing Enhancers (ESEs/ISEs)

and Exon/Intron Splicing Silencers (ESSs/ISSs). These six-nucleotide sequences are binding sites for splicing factors/repressors that regulate alternative splicing of pre-mRNAs. The presence of these regulatory elements marks the boundaries of exons (Cartegni et al., 2002). ESEs/ISEs act as binding sites for splicing factors such as SR proteins SRSF1 and SC35 while ESSs/ISSs act as binding sites for splicing repressors such as DAPAZ1 and hnRNPA1. Disruption of one or more cis-acting elements, or modulation of one or more proteins that bind to them, results in either exon skipping or exon inclusion. Exon skipping is the phenomenon in which alternative splicing of pre-mRNA results in exclusion of one or more exons from mature mRNA. Alternative splicing is beneficial when multiple proteins are correctly produced from the same pre-mRNA transcript. However, abnormal alternative splicing is implicated in diseases such as cystic fibrosis and spinal muscular atrophy (reviewed in Faustino and Cooper, 2003; Black, 2000).

Pre-mRNA processing factors localize in nuclear speckles, which are dynamic structures located in the interchromatin regions of mammalian nuclei (reviewed in Spector and Lamond, 2010). Proteomic analysis of nuclear speckles revealed the presence of at least 180 known proteins, of which many are involved in pre-mRNA processing (Mintz et al., 1999; Saitoh et al., 2004). Nuclear speckles do not contain DNA but are located in close proximity with active transcription sites and form euchromatic neighborhoods (Haung and Spector, 1991; Thiry, 1995; Spector and Lamond, 2010). The size and shape of nuclear speckles largely depends on various metabolic and environmental factors including levels of gene expression. Live cell microscopy showed formation of rounded and enlarged speckles when gene expression is inhibited suggesting that nuclear speckles act as storage/assembly/modification sites for splicing factors (Spector and Lamond 2010). Active genes are present at the periphery of nuclear speckles and polyA⁺ RNA

(but not DNA) localizes to nuclear speckles (Carter et al. 1991; Huang et al. 1994; Visa et al. 1993; reviewed in Thiry 1995). This suggests that some polyadenylated RNAs are enriched in nuclear speckles after they are transcribed. Some of these RNAs are noncoding RNAs. MALAT1 is one example of a long nuclear retained noncoding RNA that is enriched at nuclear speckles and helps in recruitment of splicing factors to transcription sites and in alternative splicing by regulating SF2/ASF phosphorylation (Hutchinson et al. 2007; Bernard et al. 2010; Tripathi et al. 2010).

Splicing of pre-mRNA transcripts occurs co-transcriptionally. The largest subunit of RNA polymerase II has a unique C terminal domain that acts as a docking site for splicing factors as well as other RNA processing factors, and hence promotes co-transcriptional loading of pre-mRNA processing factors onto nascent transcripts (Bentley, 2005; Reed, 2002; Misteli et al., 1997; Misteli and Spector 1998). Live cell imaging of cells transfected with GFP-SF2/ASF in BTK-IB cells show recruitment of GFP-SF2/ASF to the transcription site of BK virus early genes. A trail of splicing factors was observed from adjacent nuclear speckles to the transcription sites after 15-20 min of transcription activation (Misteli et al., 1997). Moreover, Misteli et al (1997) showed that the transcriptionally active genes and their pre-mRNA are located at the periphery of nuclear speckles (Misteli et al., 1997). The same was shown by another group that BrUTP labeling (which marks transcription sites) is not associated with nuclear speckles during short incubations. However, longer incubations with BrUTP marked the localization of interchromatin granule-associated zones (Cmarko et al., 1999). Taken together, all of this data supports the model that nuclear speckles supply splicing factors and other RNA processing proteins to active transcription sites.

Son regulates splicing and promotes cell cycle progression

Son is among 33 novel proteins identified in the proteomic analysis of nuclear speckles (Saitoh et al., 2004). Son is the largest known SR protein, and it has several putative functional domains including an RS domain, a glycine rich patch (G-patch) and double stranded RNA binding domain (DSRBD). RS domain contains alternative arginine and serine amino acid residues and RS domains of different proteins interact with each other to provide protein-protein interactions between different splicing factors. Phosphorylation of RS domains is essential for their function and hence splicing activity is regulated through differential phosphorylation of RS domains (Boucher et al., 2001; Blencowe, 2000; Graveley, 2000; Smith & Valcarcel, 2000). When expressed in HeLa cells, YFP-Son co-localizes with SF2/ASF in nuclear speckles (Sharma et al., 2010; Huen et al., 2010). Son also binds to the NHR4 domain of the leukemogenic protein AML1-ETO and is important for cell growth (Ahn et al., 2008). One-third of Son's amino acid sequence consists of novel repetitive sequence motifs of unknown function that are unique to Son as shown in Figure 1 (Saitoh et al., 2004). Depletion of Son in HeLa cells alters nuclear speckle organization of splicing factors SF2/ASF, SC35 and U1-70K that reorganized to doughnut-shape structures in the absence of Son (Sharma et al., 2010). The unique repeats in Son were necessary to rescue the nuclear speckle organization (Sharma et al., 2010). Just as the repeated heptad sequence in the C-terminal domain of the largest subunit of RNA polymerase II serves as a platform for dynamic recruitment of proteins involved in co-transcriptional processing of nascent transcripts, we speculate that the unique repeats in Son act as docking sites for other proteins (Sharma et al, 2010; Sharma et al., 2011).

Since Son co-localizes to nuclear speckles with splicing factors such as SF2/ASF, SC35 and small nuclear RNAs (snRNAs), Son is most likely a pre-mRNA splicing factor. Three separate studies confirmed the role of Son in accurate splicing of human mRNAs and suggest that Son plays an important role in regulating the expression of genes involved in maintaining genome stability and cell cycle progression and pluripotency (Ahn et al., 2011; Sharma et al., 2011; Lu et al 2013).

Figure 1: Motif structure of Son.

The amino terminus of Son contains six tandem repeat motifs between amino acids 334 and 1493. The carboxy-terminus of Son contains multiple domains such as a RS domain, a G-patch and a DS RNA binding domain (Source: Sharma et al., 2011).



In the first study (Ahn et al., 2011), microarray analysis in Son depleted HeLa cells altered the expression of 659 transcripts by more than 1.45-fold. Many of the affected transcripts fall into pathways and functional categories such as apoptosis, cell cycle, cancer, DNA replication/recombination/repair and amino acid metabolism. Most of the downregulated transcripts are involved in cell cycle and DNA replication/recombination/repair and upregulated transcripts belonged to the categories of cell signaling, cell death/survival and molecular transport (Ahn et al., 2011). Validation of the microarray data performed by UV crosslinking and immunoprecipitation followed by RT-PCR showed that Son is associated with mRNAs of downregulated genes such as TUBG1, KATNB1, TUBGCP2, PCNT, AKT1 and AURKB (Ahn et al., 2011).

In the second study (Sharma et al., 2011), exon array analysis of Son depleted HeLa cells showed a 2-fold decrease in expression of 568 genes and a 2-fold increase in expression of 359 genes. The affected genes fall into several biochemical pathways such as apoptosis, cell cycle, integrin mediated cell adhesion, smooth muscle contraction and G protein signaling (Sharma et al., 2011). Validation of exon array data performed by quantitative RT-PCR using primer sets within a single exon, for upregulated genes (due to Son depletion) namely GEMIN5, JL1A, TNFRSF21, CCN61 and CYP1B1 showed 2-2.5 fold up regulation. Similarly, validation of down regulated genes (due to Son depletion) such as CDK5 and TNCC1 showed nearly 50% and 60% reduction in transcript levels respectively (Sharma et al., 2011).

Exon array data performed by Sharma et al., (2011) additionally showed altered splicing (either exon inclusion or exclusion) in 1061 genes and changes in 2067 splicing events. These results suggest that Son plays a role in splice site selection during splicing of many transcripts (Sharma et al., 2011). The down regulated genes due to Son depletion such as TUBG1, KATNB1,

TUBGCP2, AURKB, PCNT, AKT1, RAD23A and FANCG were tested for splicing defects with RT-PCR using primers targeting two adjacent exons. Interestingly, intron retention was observed, indicating Son is required for accurate intron removal in a subset of transcripts (Ahn et al., 2011). Further studies on splice site selection accuracy in TUBG1 showed that Son depletion results in improper removal of multiple introns indicating that Son acts as a coactivator in constitutive splicing to avoid intron retention. Additionally, Son-dependent genes were predicted (using ESEfinder) to contain weak 5' and 3' splice sites. Moreover, some splice sites were predicted as 'dual-specificity splice sites' that can act as both 5' and 3' splice sites and thereby baffle the splicing factors involved in splice site selection. When the weak splice sites were mutated to become strengthened, they did not depend on Son for accurate splicing, suggesting that Son is required only when transcripts have weak splice sites (Ahn et al., 2011). To determine if Son is required in accurate splicing of all genes consisting of weak splice sites, Ahn et al. (2011) have mutated the splice site of TUBA1B minigene, which is a Son-independent gene, to weaken it. This mutated TUBA1B minigene did not show Son dependency for proper splicing indicating that Son is essential for proper splicing of only a subset of genes (Ahn et al., 2011). Furthermore, UV crosslinking and immunoprecipitation (CLIP) studies revealed that Son is physically associated with the mRNAs that were down regulated following Son depletion, suggesting a direct role for Son in splicing. Minigene assays (using the minigene reporter constructs to study intron retention *in vivo*) for TUBG1 pre-mRNA containing exon7-intron7-exon8 cassette or exon8-intron8-exon9 cassette showed that intron removal is directly dependent on absence or presence of Son (Ahn et al., 2011). When functional domains of Son such as the G-patch, the RS domain, or the DSRM were either deleted or cloned into expression vectors in various combinations, it was observed that the RS domain and G-patch were important for

proper splicing of TUBG1 pre-mRNA, and the C-terminal region containing an RS domain, G-patch and DSRM rescued splicing to a significant extent (Ahn et al., 2011).

Validation of the exon array data from Sharma et al. (2011) by RT-PCR showed altered splicing in Son-depleted cells for mRNAs coding for chromatin modifying enzymes HDAC6, ADA and SetD8. Exons 27 and 28 were skipped in HDAC6 mRNA, exon 2 was skipped in SetD8 mRNA and exon 9 was skipped in ADA mRNA, indicating that Son maintains proper splice site selection for all three transcripts (Sharma et al., 2011). Interestingly, Sharma et al., (2011) also showed that Son depletion resulted in skipping of exon 6 in stably integrated rat beta-tropomyosin (BTM) minigene reporter transcripts in HeLa cells. This suggested that Son is involved in regulating alternative splicing of BTM, and that minigene constructs can be useful tools for investigating splicing mechanisms *in situ*. Given that Son impacts all different types of alternative splicing mechanisms, it is important to know if the mechanisms for such regulation have any common RNA elements or if they involve common co-factors. Ahn et al., has already investigated the importance of Son for proper intron removal in a subset of transcripts. Here I propose to construct an HDAC6 reporter minigene to further investigate Son-dependent regulation of exon skipping *in situ*.

In the third study (Lu et al., 2013), Son's role in maintaining pluripotency was described. Son depletion in human Embryonic Stem Cells (hESCs) results in loss of pluripotency and cell death. Genome-wide RNA profiling in Son depleted hESCs identified a set of 1,994 introns from 1,127 genes that had splicing defects following Son depletion. OCT4, PRDM14, E4F1 and MED24 are some of the genes associated with pluripotency that showed splicing defects due to Son depletion (Lu et al., 2013).

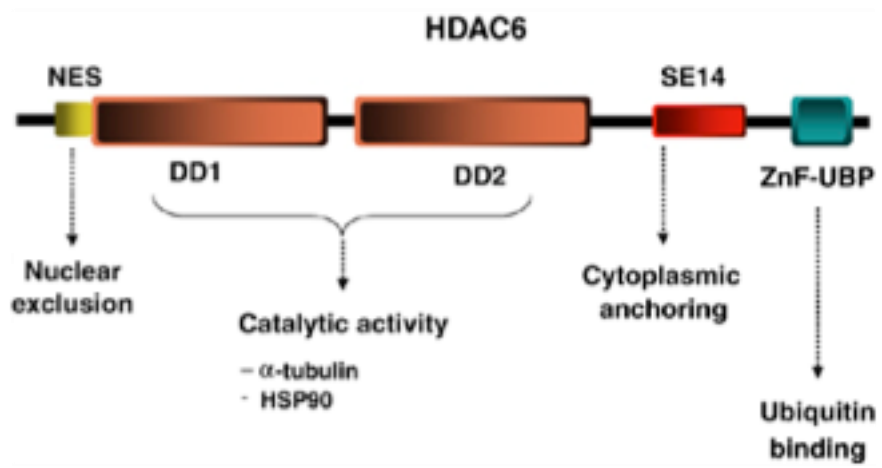
Histone deacetylase 6:

Histone deacetylases (HDACs) deacetylate the nucleosomal histone tails at conserved lysine residues. Transcription is regulated by the acetylation and deacetylation status of chromatin making HDACs important in gene regulation. HDACs are also capable of deacetylating lysine residues of transcription factors and thereby regulate their DNA binding ability. Eighteen mammalian HDACs were identified and classified into three classes I, II (A and B) and III. HDAC6 belongs to class IIB deacetylases and is a cytoplasmic protein (reviewed in Verdin et al., 2003).

The gene encoding HDAC6 is 29,894 basepairs in length and is comprised of 29 exons. It lies on X chromosome at position p11.23. The mature HDAC6 transcript is 4089 nucleotides and encodes a protein of 1215 amino acids. A naturally occurring splice variant, hHDAC6p114, was recently observed in A549 cells; this variant lacks the first 152 amino acids in its N-terminus (Zhuang et al., 2010). Among all HDACs, HDAC6 is the only HDAC that contains two functional histone deacetylase catalytic (CAT) domains and a C-terminal BUZ (binding-of-ubiquitin zinc finger) domain (Figure 2; Hook et al., 2002). The two catalytic histone deacetylase domains function in deacetylating α -tubulin present in microtubules, which play a key role in cell motility (Hubbert et al., 2002). The HDAC6 BUZ-domain (amino acids 1134-1192) is encoded by, 174 nucleotides present in exons 27 and 28 of HDAC6 mRNA. When Son is depleted in

Figure 2: Motif structure of HDAC6.

HDAC6 consists of a nuclear exclusion signal domain, two catalytic deacetylase domains, a cytoplasmic anchoring signal domain and a C-terminal BUZ-domain. (*Source: Boyault et al., 2007*).



HeLa cells, exons 27 and 28 of HDAC6 pre-mRNA are skipped (Sharma et al, 2011), presumably deleting the BUZ domain under these conditions.

The BUZ-domain of HDAC6 associates with polyubiquitin and thus with ubiquitinated misfolded proteins (Kawaguchi et al., 2003). Under normal conditions, misfolded proteins are degraded by proteasomes. Under stress, formation of misfolded proteins increases and becomes toxic to the cell (Ward et al., 1995). In several human disorders, the accumulation of misfolded proteins leads to cell death. One mechanism to degrade the misfolded proteins is formation of aggresomes (Kawaguchi et al., 2003). A model suggests that HDAC6 interacts with dynein motor proteins as well as ubiquitinated misfolded proteins and directs them to the microtubule-organizing center (MTOC) via microtubules, where aggresomes are formed (Kawaguchi et al., 2003). Once aggresomes are formed, the misfolded proteins are degraded by the local proteases and autophagy proteins. Mutant HDAC6 lacking either catalytic deacetylase domains or BUZ-domain could not rescue aggresome formation, suggesting the importance of deacetylase domains and BUZ-domains in aggresome formation (Kawaguchi et al., 2003).

Minigene assays

Several genes express more than one type of mRNAs through alternative splicing. Exon recognition plays an important role in alternative splicing. Several factors influence the recognition of exon such as strength of splice sites, sizes of alternatively spliced exons or the introns on either side, and the presence of secondary structure (Cooper, 2005). Reporter minigene assays involve construction of a reporter plasmid containing a small portion of genomic DNA including the alternatively spliced exon(s), flanking introns and exons. These reporter plasmids are then used in *in vivo* analysis of *cis*-regulatory elements (such as cryptic

splice sites, ESEs and ESSs) and *trans*-acting factors such as splicing factors and splicing repressors (Cooper, 2005). Since endogenous transcripts cannot be visualized easily *in situ* and the large size of the endogenous genes and transcripts makes it difficult to analyze the binding partners and protein-RNA interactions, minigene assays are important tools to analyze the proteins recruited to the transcription/splicing sites of the minigene. Moreover, inducing silent mutations in the minigene can help us determine ESEs/ESSs that are essential for proper splicing of the transcripts and can also determine the binding regions for several splicing factors and splicing repressors (Cooper, 2005, Ahn et al., 2011).

CHAPTER 2: HYPOTHESES AND SPECIFIC AIMS

HYPOTHESES

Hypothesis 1: To construct an HDAC6 minigene reporter

Sharma et al (2011) showed that Son depletion causes alternative splicing of endogenous HDAC6 and exogenous BTM minigene. Ahn et al. (2011) showed that Son is required for constitutive splicing of both endogenous and exogenous TUBG1. Based on the above studies on requirement of Son in splicing, I hypothesized that inclusion of exons 27 and 28 in HDAC6 minigene reporter transcripts requires Son.

Hypothesis 2: To analyze splicing of HDAC6 minigene reporter transcripts in Son-depleted cells

Based on the work performed by Sharma et al. (2011) and Ahn et al. (2011), as well as on rescue experiments employing Son and its deletion mutants, I hypothesized that the full length Son and its deletion mutant containing the C-terminal RS domain can rescue the exon skipping in HDAC6 transcripts. Millevoi et al (2010) showed that hnRNPA1 and hnRNP H/F are involved in the alternative splicing of BRCA1 transcripts. They found that single base-pair substitution occurred in the gene and resulted in creation of binding sites for the two splicing regulators. Sharma et al (2011) performed *in situ* studies to show that splicing factors such as SF2/ASF and U1-70K are present at the BTM minigene transcription site both before and after Son depletion. Recruitment of splicing factors to the co-transcriptional splicing sites results in accurate splice site selection followed by spliceosome assembly, whereas recruitment of hnRNPs results in formation of nonproductive spliceosomes or arrests the spliceosome assembly (Erkelenz et al.,

2013). Based on the experimental results from the above studies and previous work in our lab on alternative splicing of HDAC6 in the absence of Son, I hypothesized that Son has a role in blocking hnRNPs from binding to nascent pre-mRNAs and thereby regulates alternative splicing.

SPECIFIC AIMS

Aim 1: To construct an HDAC6 minigene reporter

I designed a minigene reporter system to investigate the mechanism by which Son regulates inclusion of exons 27 and 28 in HDAC6 mRNA.

Aim 1a: I generated a HDAC6 minigene cassette as a model minigene reporter system to study the mechanism of splicing regulation by Son. The HDAC6 minigene cassette, consisting of genomic DNA encoding E26-I26-E27-I27-E28-I28-E29 (where E represents exon and I represents intron), was cloned into pcDNA3.1. This reporter plasmid was transfected into HeLa cells to test whether the exons 27 and 28 are skipped following Son depletion. RNA was extracted from transfected cells, and RT-PCR was performed using primers specific to exogenous minigene transcripts. Endogenous HDAC6 transcripts served as a positive control for exon skipping after Son depletion.

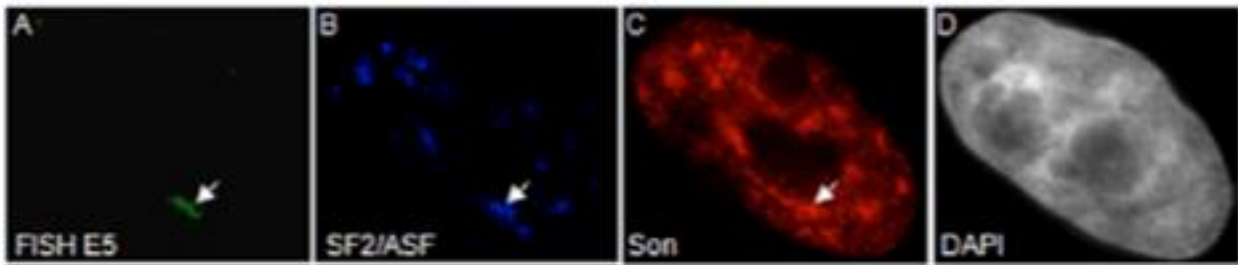
Aim 1b: After confirming that the transiently transfected HDAC6 minigene reporter shows skipping of exon 27 and 28, I stably transfected HeLa cells with the HDAC6 minigene reporter plasmid and screened clones that are resistant to the drug G418 which is an analog to neomycin. The selected clones were expanded for primary and secondary screening. Screening was performed by extracting RNA from each separate clone for RT-PCR analysis to detect

exogenous HDAC6 transcripts using an upstream primer targeting T7 sequences encoded by the plasmid and a downstream primer targeting exon 29. The stable cell lines expressing exogenous HDAC6 minigene reporter transcripts were then characterized to verify that skipping of exons 27 and 28 occurs after Son depletion.

Aim 1c: RNA-FISH using the fluorescent tagged-oligos designed either at T7 promoter region of exogenous HDAC6 minigene or in exon 26 was performed in HeLa HDAC6 clones, HeLa HDAC6 stable populations and HeLa cells transiently transfected with HDAC6 plasmid to identify the HeLa HDAC6 clones containing a reporter locus (ideally it is expected to be detected as a single dot in each nucleus as shown for BTM cell line in Fig. 3).

Figure 3: SRSF1 and Son are recruited to the exogenous BTM minigene transcription site.

A. RNA-FISH using a fluorescently conjugated probe targeting exon 5 of rat beta-tropomyosin labeled the BTM reporter transcription site. B, C. Immunofluorescence localization of SRSF1 and Son in BTM HeLa cells. Arrows indicate the transcription site of the BTM reporter minigene (Source: Sharma et al., 2011).



Aim 2: To analyze splicing of HDAC6 minigene reporter transcripts in Son-depleted cells

The first series of experiments determined which regions of Son are necessary for maintaining correct splicing of HDAC6 transcripts. To achieve this, I transfected HEK 293 cells with siRNA-refractory (siR) constructs including full length YFP-SiR-Son and three deletion mutants of Son (YFP-SiR-Son-1-2008, YFP-SiR-Son-1-1493 and YFP-SiR-Son-1-332) and selected stable clones expressing the constructs using G418. The stable cell lines were transfected with two different siRNA duplexes and a non-targeting luciferase gene siRNA (control) against Son followed by RT-PCR using primers sets targeting exon 26-29 region of endogenous HDAC6. The overexpression of siR-Son constructs and knockdown of endogenous Son were validated by qRT-PCR using primer sets specific to exogenous and endogenous Son. These experiments help us determine which deletion mutants can rescue exon skipping of HDAC6 and hence can directly implicate Son in regulation of splicing.

The next series of experiments aims to observe alternative splicing of HDAC6 minigene *in situ* and determine the role of Son in recruiting splicing enhancers/repressors to nascent HDAC6 transcripts. In the attempt to visualize the transcription loci of HDAC6 minigene (as in specific aim 1C), the experiments were designed to perform immunofluorescence with antibodies against Son and SF2/ASF along with RNA-FISH with T7 or E26 probes.

CHAPTER 3: MATERIALS AND METHODS

HDAC6 subcloning

Human HDAC6 genomic DNA containing exons 26, 27, 28, 29 and the intervening introns was selected for minigene reporter system construction as Son depletion causes exclusion of exons 27 and 28 in HDAC6 transcripts. BAC clone RP11-416B14 containing HDAC6 gene was purchased from Children's Hospital Oakland Research Institute (CHORI; Oakland, CA). The BAC clone library was initially constructed by isolating genomic DNA from male human blood cells. HDAC6 minigene was then amplified using 5' CAAGAATCGGGCTTCTCTGA 3' present in intron 25 region and 5' TCTCCACCTGCTCAAAGTCA 3' present in intron 29 region as forward and reverse primers respectively. The amplified PCR product of size 1,387 bp was cloned into PCR2.1 vector (TA cloning kit, Invitrogen). The positive clone from TA cloning was used as a template to amplify HDAC6 minigene. Primers were designed to amplify the region containing exon 26 through 29 to avoid unnecessary confusion about splice site selection. The forward primer 5'-GGGGGATCCGGGGCCTCAGAATCTCAG-3' contains a flanking BamH1 restriction site (underlined) and the reverse primer 5'-GGGGCTCGAGAACAGCTTGTACTTTATT-3' contains a flanking Xho1 restriction site (underlined). For PCR, input DNA (100 ng), forward and reverse primers (100 ng each), magnesium chloride (50 mM, Finnzymes, PA), pfu Ultra Hotstart DNA Polymerase (Aligent Technologies, CA), 10X pfu Ultra Hotstart DNA polymerase buffer, dNTPs (10mM, Aligent Technologies, CA) and Molecular Biology Grade water (5 Prime, MD) were used. pcDNA3.1 was used as the vector to subclone HDAC6 minigene as it contains antibiotic resistant genes for

both ampicillin and neomycin which allows its selection in both bacterial and mammalian cells. Empty pcDNA3.1-V5-His-A (Invitrogen, CA) and HDAC6 minigene amplified from TA-HDAC6 clone were separately digested with BamH1 and Xho1. Gel extraction (Cat. No. 20021; Qiagen, MD) was performed for both insert (HDAC6 minigene) and double digested pcDNA3.1. Ligation was performed using T4 DNA ligase (Invitrogen, CA). The ligation reaction was transformed to DH5 α cells (Invitrogen, CA) following the manufacturer's protocol. Bacterial colonies were screened for the presence of the vector containing HDAC6 minigene (insert). Plasmid DNA was isolated and digested with different enzymes to make sure the DNA bands of expected size were obtained. Plasmid DNA isolated from the three positive bacterial clones was sequenced to ensure no mutations were present.

Cell culture

HeLa cells obtained from Dr. David Spector (CSHL, NY) and HEK 293 cells (ATCC, VA) were grown and maintained in 100 mm X 20 mm culture dishes in Dulbecco's Modified Eagle Medium (DMEM; Hyclone, Thermochemical, Utah) with 10% fetal bovine serum (FBS; Hyclone, Thermochemical, Utah) and 1% penicillin/streptomycin (Invitrogen, CA). All cells were maintained in a humidified incubator (37°C) in the presence of 5% carbon dioxide. The cells were passaged after reaching 80% confluency. To passage cells, the cells were briefly washed 3 times with 1X phosphate buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na₂HPO₄; 1.47 mM KH₂PO₄, pH 7.4) and were treated with 2ml 0.25% trypsin/EDTA (Hyclone, Thermochemical, Utah) for 2 min at 37°C. 5 ml DMEM containing 10% FBS was added to inactivate trypsin and the cells were collected in to 15 ml conical tubes, spun for 2 min at 1500 rpm. Supernatant was aspirated and the pellet of cells was resuspended in fresh DMEM

supplemented with FBS and antibiotics, either plated in 100 X 20mm dishes for passage or counted and plated in 6 well dishes.

Counting and plating cells

To plate cells for Son depletion and for RNA-FISH experiments, the cells were collected from 100 X 20mm plates, centrifuged and resuspended as described in the cell culture section above. 10 μ l of cell suspension was loaded onto each side of the Bright-Line Hemocytometer and the cells were counted and averaged to find the number of cells per 1 ml. Cells were diluted with DMEM + 10% FBS (for Son depletion) or with DMEM + 10% FBS and 1% penicillin/streptomycin (for RNA-FISH) accordingly to get a final concentration of 1 X 10⁵ (for Son depletion) or 1.5 X 10⁵ (for RNA-FISH).

Freezing and thawing cells

To freeze the cells, the cells were expanded to reach 80% confluency in 100 mm dishes and washed with 1X PBS. After washing, the cells were trypsinized, collected in to 15 ml conical tubes and centrifuge at 1500 rpm for 2 minutes. The supernatant was aspirated and 1 ml of ice-cold freezing medium (95% FBS and 5% DMSO (Cat. No. D2650, Sigma, MO)) was added, mixed and 0.5 ml was added to each cryovial. The cryovials were then placed in Styrofoam racks (to slowdown the freezing process) and transferred to -80°C freezer.

To thaw, the vial was placed in 37°C water bath briefly, 5 ml DMEM +10% FBS +1% pen/strep was added and transferred to a 15 ml conical tube. The tube was centrifuged for 2 min at 1500 rpm and the supernatant was aspirated. To the pellet, 10 ml fresh medium was added and plated in 100 mm dish and placed in 37°C humidified incubator supplemented with 5% CO₂. Thawing

process was carried out as fast as possible as DMSO present in the freezing medium is harmful to cells at higher temperatures.

Transfections

HeLa cells were cultured (~80% confluency), trypsinized and collected as described previously. The cells were resuspended in 1 ml of medium. Salmon sperm DNA was used along with HDAC6 reporter plasmid to ensure equal distribution of the plasmid to cells. 2 μ l of salmon sperm DNA (20 μ g/ μ l), 2 μ g of HDAC6 reporter plasmid and 250 μ l cell suspension were added to a 4 mm gap cuvette. Electroporation was performed by using GenePulser X Cell (250V, 950 μ F and 4 Ω ; BioRad, CA). Transient transfections were optimized in HeLa cells to determine the optimal amount of DNA as well as the post-transfection time required to detect expression of reporter transcripts. For stable transfections, G418 (1mg/ml) was added to the medium for selection.

Son depletion

Son depletion was achieved by RNAi using Son siRNA duplexes (si1 catalog # J-012983-05 or si4 J-012983-08; Dharmacon RNA Technologies). A non-targeting siRNA duplex for luciferase (catalog no. D-001210-02; Dharmacon RNA Technologies) was used as a control to ensure that the changes observed are not simply due to activation of RNAi machinery. Son depletion was validated by immunofluorescence with antibodies against Son and qRT-PCR using primer sets targeting Son mRNA, standardized to GAPDH. The quantitative results (ct values) of GAPDH

qRT-PCR was subtracted from Son qRT-PCR results to determine Δct . $\Delta\Delta\text{ct}$ was calculated by subtracting the effect of control siRNA. Fold change was then determined by calculating $2^{-\Delta\Delta\text{ct}}$. A graph is then plotted between the fold change and samples such as control, siRNA 1 and siRNA 4.

Son depletion in HEK293 cells stably expressing siRNA-refractory YFP-Son constructs was achieved by using siRNA duplex 4, as the deletion mutants contain silent mutations in the region of Son mRNA that is targeted by siRNA duplex 4. siRNA duplex 1 was also used to deplete both exogenous and endogenous Son in control rescue experiments. A luciferase-targeting control siRNA duplex was used as control to ensure that the changes observed are not simply due to the activation of RNAi machinery. Overexpression of siRNA refractory YFP-Son constructs and endogenous Son depletion was confirmed by qRT-PCR using primer sets targeting exogenous, endogenous Son and GAPDH.

RNA-FISH

RNA-FISH was performed using the T7 probe that is specific to exogenous HDAC6 minigene transcripts and/or E26 probe which binds to exon 26 region of both exogenous and endogenous HDAC6 mRNA. All the positive HeLa HDAC6 clones were screened to identify those containing a single reporter locus. The cells were fixed using 2% formaldehyde, washed three times 5 min each with 1XPBS and permeabilized using Triton X-100 in the presence of 250mM vanadyl-ribonucleoside complex (VRC) for 8 min to prevent RNase activity. Further, the cells were washed with 1X PBS two times 5 min each and blocked using RNase free 0.5% BSA in 1X PBS for 15 min. The cells were then washed in 1X SSC for 5 min. The hybridization mixture was prepared by adding RNA-FISH probe(s), 2X SSC, 1mg/ml of tRNA, 10% dextran sulfate,

and 25% formamide. Hybridization was performed by adding 20 μ l hybridization mixture to clean glass slides and inverting the coverslips (cells facing down) and then the coverslips were sealed with rubber cement on all four sides. Glass slides were incubated at 37°C for three hours, in a humidified chamber. The cells were washed in 2X SSC and 25% formamide for 30 min at 37°C followed by a 30 min wash in 2X SSC at room temperature. The cells were then briefly washed in 2X SSC and then processed for immunofluorescence. Microscopy was performed on a Deltavision RT microscope, and images were collected and processed using SoftWorx software.

Stable cell line construction

To construct HeLa HDAC6 stable cell line, HeLa cells were transfected with HDAC6 subclone as described below. The next day, cells were split to 10 plates (100 X 100 mm) to make sure the cells are separated enough to form individual colonies. Third day, Geneticin or G418 (1 mg/ml) is added to the cells. G418 is an analog to neomycin sulfate and it blocks protein synthesis by interfering with the function of 80S ribosomes. Neo^r gene present in HDAC6 plasmid produces aminoglycoside 3'-phosphotransferase, APT 3' II which is resistant to G418. The cells were then allowed to grow for 2-3 weeks within which the cells that integrate the plasmid into their genome and make APT 3' II do survive and develop their own colonies while untransfected cells and cells in which plasmid does not integrate into the genome die. The colonies were picked later into 24 well dishes and allowed to grow until the wells were confluent. For primary screening of clones stably expressing HDAC reporter transcripts, RNA was extracted from the 96 clones followed by DNase treatment and RT-PCR using T7 forward primer and E29 reverse primer . Positive clones from primary screening were expanded and screened a second time to confirm the expression of minigene transcripts. The positives from secondary screening were expanded and frozen down.

Immunofluorescence

Coverslips were washed thoroughly in one part nitric acid and two parts hydrochloric acid for two hours, rinsed in distilled water continuously until the pH returns to 7.0. The coverslips were then stored in 70% ethanol and flamed before using to remove ethanol. Cells grown on coverslips were washed once with 1X PBS and fixed with 2% paraformaldehyde (in 1X PBS) for 15 min followed by three washes with 1X PBS 5 min each. The cells were then permeabilized for 5 min with 0.2% TritonX-100 and washed three times with 0.5% normal goat serum (NGS, Gibco) in 1X PBS. Washes with normal goat serum help in blocking the proteins. The coverslips were then placed with the cell side up in a humidified chamber prepared by putting a wet filter paper in a 15 cm plate. 40µl of primary antibodies diluted in 0.5% NGS-1X PBS were added to the cells and incubated for one hour at room temperature. Rabbit polyclonal antibodies were developed (Covance; Denver, PA) against the C-terminal peptide sequence SPNKKHAKATAATV of Son (Wu13; 1:100), and against the N-terminal peptide sequence CEESESKTKSH of Son (Wu14; 1:1000) and monoclonal anti-SF2/ASF AK103 (1:2500; provided by A. Krainer, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Coverslips were placed back into the appropriate dishes and washed three times with 0.5% NGS in 1X PBS. 40µl of secondary antibodies diluted in 0.5% NGS -1X PBS were added to cells placed in humidified chamber cell side up. Fluorescently- conjugated secondary antibodies donkey anti-mouse (Cy5) and donkey anti-rabbit (FITC or TxRed) proteins (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) were used. After one hour incubation with secondary antibodies, the coverslips were placed back into the appropriate dishes and washed two times with 0.5% NGS in 1X PBS for 5 min each. The cells were then washed for 5 min in 4', 6-diamidino-2-phenylindole (DAPI; 10 µg/ml diluted 1:1000 in 1X PBS) to stain DNA and washed once with

1X PBS. To mount the coverslips, either anti-fade mounting medium (p-phenylenediamine, glycerol, pH. 8.0 - 9.0) or N-propyl-gallate mounting medium (for CY5-conjugated antibodies) was used and the coverslips were placed cell side down on clean microscope glass slides with 8-10 μ l mounting medium. Excess mounting medium was removed by using a filter paper and the edges of coverslips were painted with clear nail polish. The slides were then stored in -80°C or imaged using DeltaVision RT microscope (Applied Precision).

RNA Extraction

RNA extraction was performed by using Qiagen RNeasy mini prep kit according to manufacturer instructions (Qiagen, CA). Briefly, cells cultured in 6 well dishes were trypsinized and collected by centrifuging for 5 min at 1500 rpm. The supernatant was completely aspirated and the cell pellet was resuspended in 350 μ l buffer RLT. The cell suspension was homogenized by vortexing for 30 sec and 350 μ l 70% ethanol was added. 700 μ l of the above solution was loaded into RNeasy spin column placed in a 2 ml collection tube, centrifuged for 15 sec at full speed (13,200 rpm) and the flow through was discarded. 700 μ l of buffer RW1 was added to the spin column, centrifuged for 15 sec at full speed and the flow through was discarded. The spin column was then washed 2 times with 500 μ l of buffer RPE and centrifuged (first time for 15 sec and second time for 2 min) at full speed. To elute RNA, the spin column was placed in 1.7 ml collection tube, 30 μ l RNase-free water was added and the tube was centrifuged for 1 min at 14,000 rpm in an Eppendorf microcentrifuge. An optional elution step was performed by adding 30 μ l of RNase-free water and collecting RNA in a fresh tube. Eluted RNA was stored at -80°C .

DNase treatment

The concentration of RNA extracted as described above, was measured using a Nanodrop spectrophotometer (Thermo Scientific). Buffer, Turbo DNase activation buffer (10%), 5 µg of RNA, Turbo DNase (1µl) were mixed and brought to 50 µl final volume with RNase free water. The samples were incubated at 37°C for 30 min followed by the addition of DNase inactivation reagent (10%) and incubated for 2 min at room temperature with occasional mixing. The samples were centrifuged for 1.5 min at 10,000 X g and the supernatant containing DNA-free RNA was transferred to new 1.5 ml tubes and stored at -80°C.

RT-PCR and quantitative RT-PCR

RT-PCR was performed by using qScript-One step RT-PCR system and qScript-two step RT-PCR system (Quanta Biosciences, MD Cat. No. 95047, 95073). For one-step RT-PCR, 100 ng of DNase treated RNA was used as template, 3 µM forward and reverse primers, 2X Sybrgreen buffer, 1 µl reverse transcriptase and RNase-free water were used to make 10 µl reactions. Master mix was prepared by adding appropriate amounts of SybrGreen buffer, reverse transcriptase, forward and reverse primers and water, mixed and centrifuged briefly and added to each of the Cepheid tubes. 100 ng of RNA was added to each of the tubes and centrifuged briefly. The tubes were then loaded in Cepheid one StepRT-PCR machine (Cepheid, CA). Amplification was performed for 40 cycles As exon skipping occurs in HDAC6 transcripts upon Son depletion, reverse transcription-PCR results in amplification results show more than one PCR product when certain primer pairs were used. In these cases, the RT-PCR products were applied to either 2% agarose gels or 10% native polyacrylamide gels (30% Polyacrylamide, 10µl TEMED, 10% APS, 5X TBE) at 80V for 90 min in 1X TBE. The gels were stained on shaking

platform by adding 2 μ l of 10 mg/ml ethidium bromide to 300 ml of buffer 1X TBE for 5 min before exposing to ultraviolet light.

For two-step RT-PCR, cDNA was prepared by mixing 4 μ l cDNA fast mix, 100 ng DNase-treated RNA, 1 μ l reverse transcriptase and RNase-free water to make up the reactions to 20 μ l. The samples were incubated in DNA engine DYAD thermo cycler (MJ Researchers, MA) with cycle conditions: 5 min at 22°C; 30 min at 42°C and 5 min at 85°C. cDNA was either stored at -20°C or diluted 3 fold and used as template for qPCR. qPCR was performed in 96 well plates in a qPCR machine (Applied Biosciences). For qPCR, master mixes were prepared separately for each primer set. 5 μ l of Perfecta fast mix, 3 μ M forward primer, 3 μ M reverse primer and RNase-free water were mixed per reaction to make mastermix and aliquoted to the reaction wells followed by addition of 3 μ l of diluted cDNA template. The quantitative results (ct values) of GAPDH qRT-PCR were subtracted from Son qRT-PCR results to determine Δ ct. $\Delta\Delta$ ct was calculated by subtracting the effect of control siRNA. Fold change was then determined by calculating $2^{-\Delta\Delta$ ct}.

Primer and probe design

All primers designed were checked for at least 50% GC content and melting temperature (above 45°C), The primers were checked for possibility of forming self-dimers, heterodimers and hairpin structures by using <http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>. Hairpin structures form when the melting temperature of hairpin structures suggested is close to that of the primer. Self-dimers or hetero-dimers form when the Δ G value is lower than -10 k. Furthermore, the specificity of the primer sequences were tested using <http://blast.ncbi.nlm.nih.gov/Blast.cgi> website. Nucleotide blast, Refseq mRNA, Homo sapiens

were selected to blast the primer sequence. For non-specific binding to occur both forward and reverse primer should bind to the same gene/mRNA or genes that are adjacent.

Table 1: List of Primers used for PCR/qRT-PCR:

HDAC6T7For	CTCACTATAGGGAGACCCAAG
HDAC6intr27For	GAGGAAGGGGATGGGGCGGA
HDAC6intr28For	GGTTGAGGGCTGGAGTGGGGG
HDAC6BGHFor	GGCTGGCAACTAGAAGGCAC
HDAC6BamHIfor	GGGGGATCCGGGGCCTCAGAATCTCAG
HDAC6XhoIrev	GGGGCTCGAGAACAGCTTGTACTTTATT
SonforRT	CAGAACTACGATATAAGCC
SonRevRT	GATACAACCTGACAGTTCTGC
GAPDHForRT	ATGGGGAAGGTGAAGGTCGGAG
GAPDHrevRT	CATGTAGTTGAGGTCAATGAAGG
HDAC EJ 26/27 revRT	AAATATGGCCTGATCAGT
HDAC EJ 26/29 revRT	TAGGAGAGCCTGATCAGT
HDAC EJ 28/29 revRT	TAGGAGAGCCTGGTGGTG
ADAej9/10revRT	TCATTTTTGAGCCGAATGACTGCA
ADAej8/10revRT	TCATTTTTGAGCCTCGAAGTGCAT
SETD8ej2/3revRT	AAATACGTTCTCCCCGTCGGTGCG
SETD8ej1/3revRT	AAATACGTTCTCCTCTAGCCATGG
SETD8ex1forRT	GATCCCAGGCGGTGACAGAGT
HDAC6 Ex25	CGAGCTGATCCAAACTCCTCTA
HDAC6 Ex26 F	AGGTCAGGACATGGCTGATT

HDAC6 Ex29 Rev

CCGTATTCTGGGGCTTAGTG

Table 2: List of RNA-FISH probes:

T7 TxRed,	(TxRed)AGCTTGGGTCTCCCTATAGTGAGTCGTATT
HDAC E26 TxRed,	(TxRed)CTAGTAGGTTCTCCTCTCCTGGGGCCTGAG

Microscopy

Images were collected in z-stacks using a 60X objective (1.4 numerical aperture; Olympus, Tokyo, Japan) on a DeltaVision RT microscope (Applied Precision). The raw data from z-stacks were displayed as volume projections.

Sequencing

The HDAC6 reporter minigene plasmid construct was sequenced to confirm that no mutations are present. Midipreps were prepared for three of the positive clones (2, 21 and 36). Since the size of the HDAC6 minigene is 1.3Kb, a forward primer was designed at the T7 promoter region, two forward primers were designed at a distance of 400bp each and a reverse primer was designed at the BGH signal. 1.2µg of plasmid DNA and 100 pmole primer was added and sent for sequencing (Retrogen,inc, CA). Results were analyzed using multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

CHAPTER 4: RESULTS

4.1 Construction of a HDAC6 reporter transgene system

Son was previously reported to be required for proper splicing of HDAC6 transcripts (Sharma et al., 2011). To further understand the mechanism of aberrant splicing caused when Son is depleted, I designed reporter minigene assays. HDAC6 subclone containing genomic segment encompassing exons 26-29 was constructed in pcDNA 3.1 and the positives from subcloning are shown in Figure 4. Genomic sequence for HDAC6 was amplified by PCR and subcloned into pcDNA3.1. Plasmid DNA was digested with BamH1 and Xho1, and colonies containing a 1.3 Kb insert along with a 5.5Kb band were chosen as positive clones (Fig. 4A, 4B and 4C). The positive clones 2, 21 and 36 were digested with various restriction enzyme combinations to confirm the presence of the HDAC6 minigene. Furthermore, three clones were sequenced to confirm that no mutations were introduced during PCR amplification.

4.2 Son-depleted cells show exon skipping in endogenous HDAC6 mRNA and transiently expressed HDAC6 reporter transcripts

To examine splicing changes in exogenous HDAC6 transcripts upon Son depletion, HeLa cells were transiently transfected with HDAC6 reporter minigene plasmid, followed by siRNA transfection to deplete Son. Reduction of Son transcripts was confirmed by qRT-PCR with primers targeting Son mRNA (Fig. 5A) and immunofluorescence where Son depleted individual cells show loss of speckle pattern when compared to mock and control oligo transfected cells (Fig. 5B). Son depleted samples showed a clear difference in splicing of reporter transcripts as seen by polyacrylamide gel electrophoresis of RT-PCR products. While cells treated with mock

Figure 4: Subcloning of the HDAC6 reporter minigene.

HDAC6 minigene PCR products were subcloned in to pcDNA3.1. **A, B, C:** Miniprep DNA isolated from amp-resistant bacterial colonies was double digested with Xho1 and BamH1. Gel images show positive clones containing two fragments; one corresponding to plasmid backbone (5.5 Kb) and the other to the minigene (1.3 Kb). Some of the clones contain an extra band at 6.8 Kb which is the linearized plasmid. Controls included undigested empty vector (lane 1 of Panels A, B and C), linearized empty vector (lane 2 of Panels A, B and C) and empty vector digested with each of the enzymes (lanes 13 and 14 of panel C).

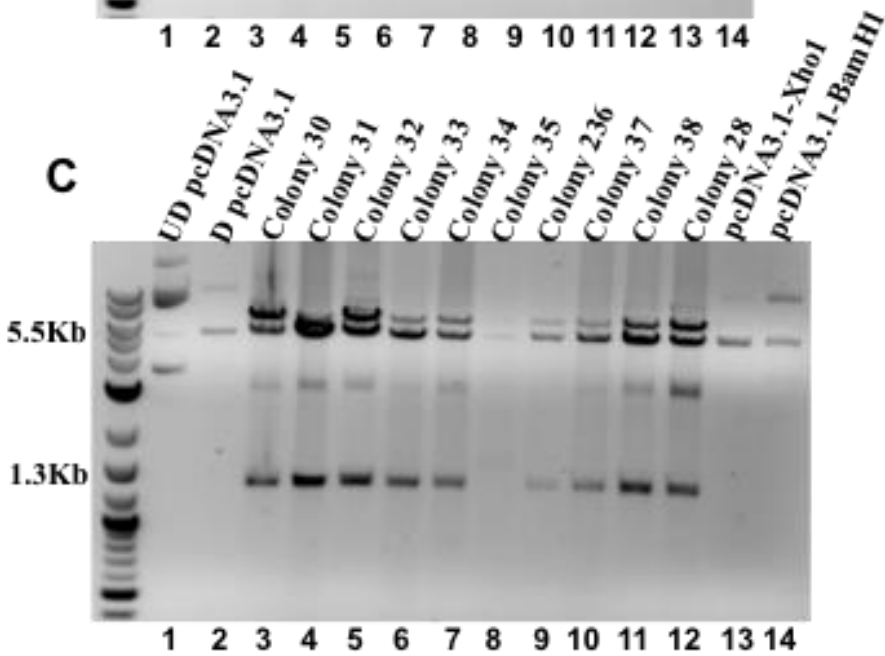
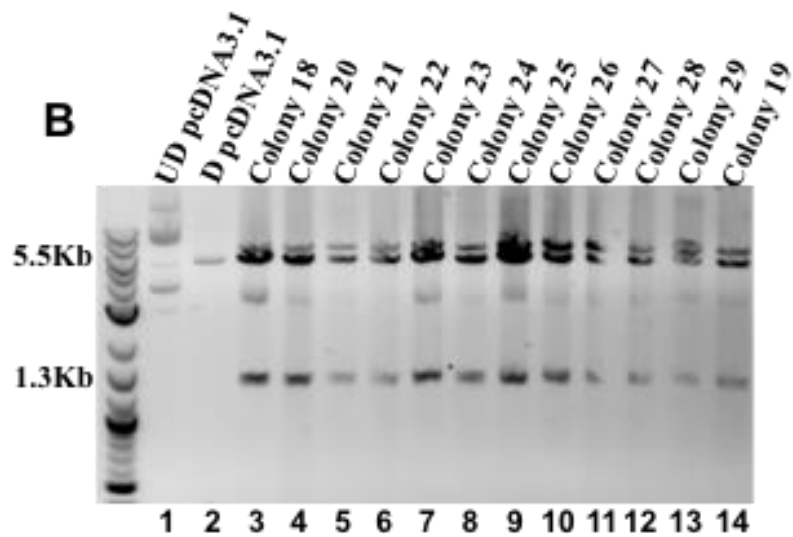
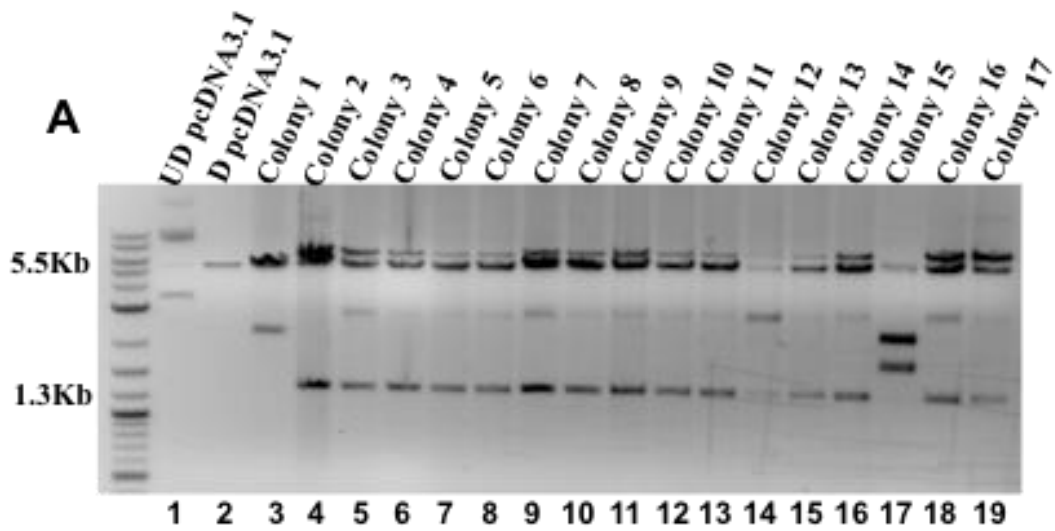
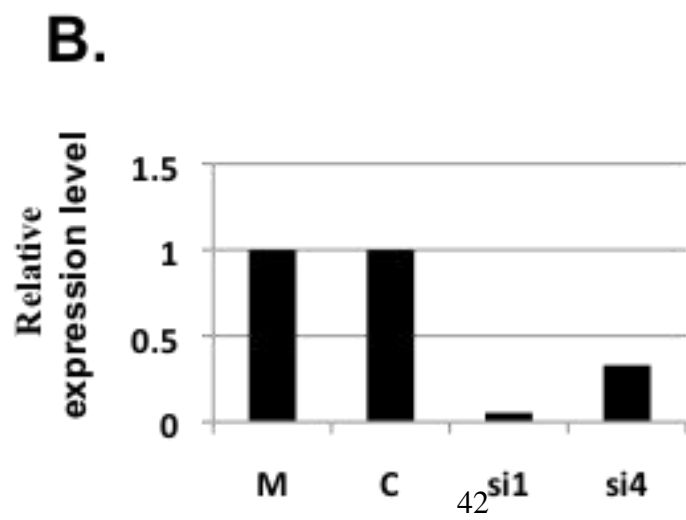
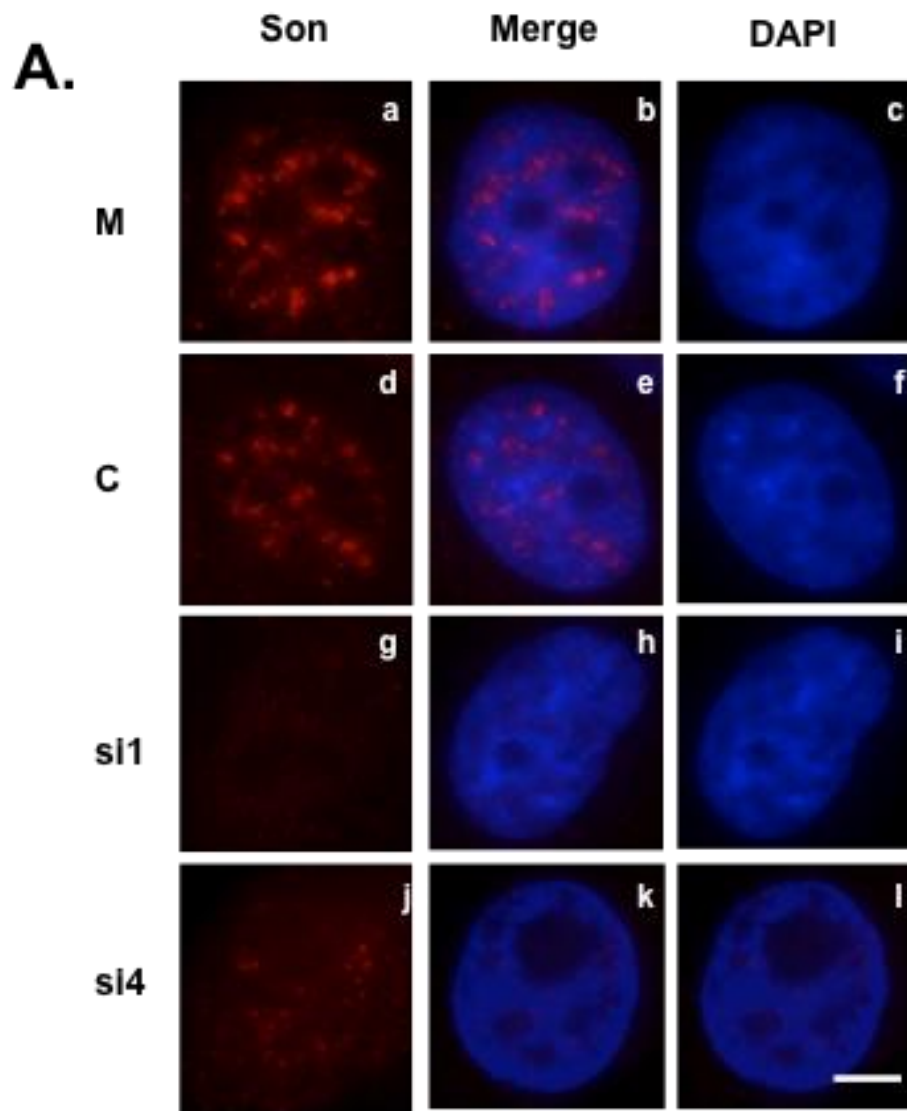
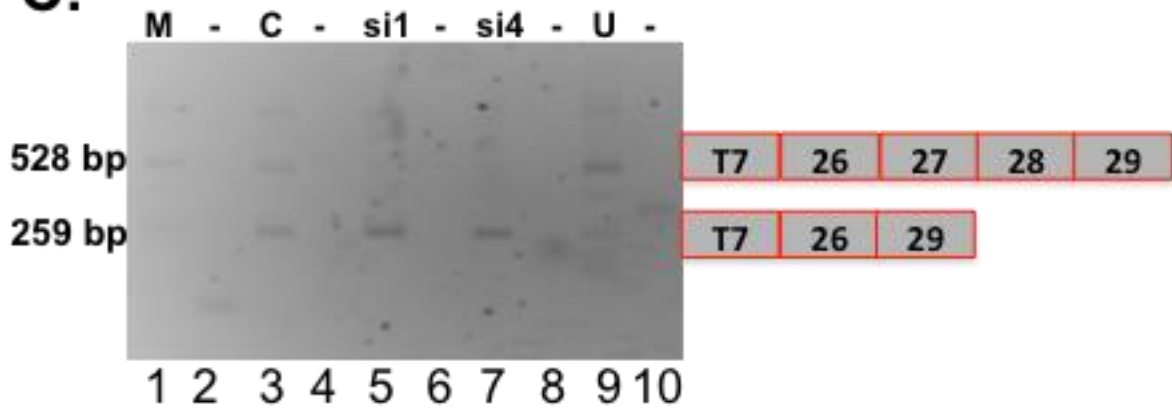


Figure 5: Skipping of exons 27 and 28 of the HDAC6 reporter minigene in Son-depleted cells.

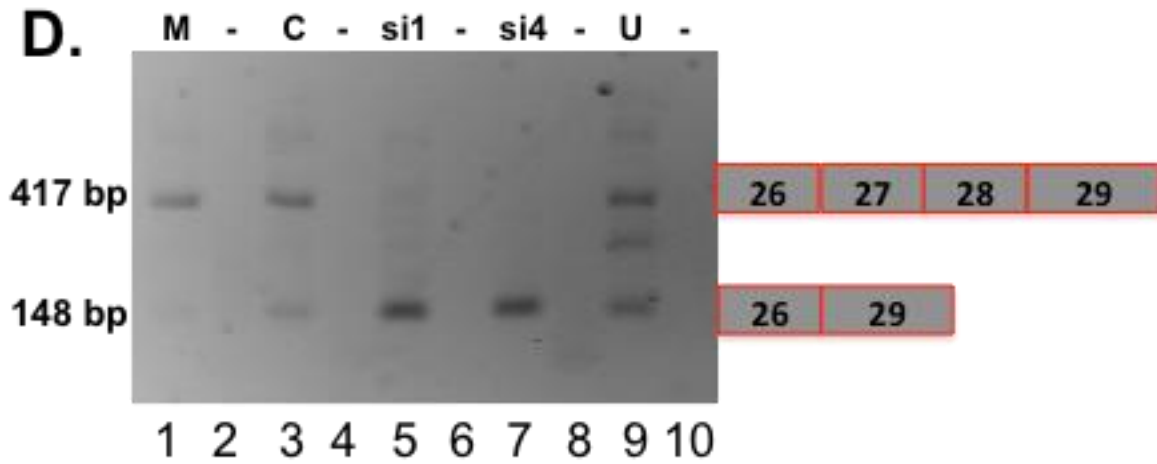
HeLa cells transiently transfected with HDAC6 reporter plasmid and Son siRNA duplexes were either processed for immunofluorescence (A) or harvested to perform qRT-PCR (B) to show Son depletion. Cells treated with Son siRNAs and immunostained with anti-Son antibodies in (A) do not show Son labeling in nuclear speckles (g and j) when compared to mock and control siRNA-treated cells (a and d). DNA was stained with DAPI (c, f, I and l). Bar = 5 μ m. qRT-PCR in (B) showed reduction of Son mRNA levels in siRNA-treated samples when compared to that of mock and control siRNA treated samples. (C) qRT-PCR performed in triplicate using primers that amplify only reporter minigene transcripts show exon skipping in Son siRNA-treated samples. Lanes labeled as “-” (2, 4, 6, 8 and 10) represent reactions in which reverse transcriptase was omitted. (D) RT-PCR performed with primers that amplify both endogenous and reporter minigene transcripts show exon skipping in siRNA-treated samples. Lanes labeled “U” represent untreated samples that were transfected with HDAC6 reporter plasmid and harvested. Lanes labeled as “-” (2, 4, 6, 8 and 10) represent reactions in which reverse transcriptase was omitted.



C.



D.



transfection or control oligo showed inclusion of exons 27-28, these exons were skipped in Son depleted cells (Fig. 5C). Splicing changes observed in exogenous HDAC6 minigene transcripts are consistent with that of endogenous HDAC6 transcripts (Fig. 5D and Sharma et al., 2011).

4.3 HeLa HDAC6 cell lines stably express HDAC6 minigene

To further investigate the mechanism of Son-mediated splicing in situ, it is important to construct cell lines stably expressing HDAC6 minigene. HeLa cells were hence transfected with HDAC6 subclone and G418 (1 mg/ml) was added to medium to select clones stably expressing HDAC6 minigene. Primary screening by RT-PCR for RNA extracted from each of the clones show unspliced, fully spliced and exon skipped products in clones 6, 19, 57, 65, 69, 79, 80 and 93 (Fig. 6A, 6B and 6C). Secondary screening was performed in which the positives from primary screening were expanded and RNA was extracted. RT-PCR for the clones mentioned above showed stable expression of HDAC6 minigene in clones 19, 57, 65, 69, 79 and 80 (Fig. 7A, 7B and 7C).

4.4 Son-depleted cells show exon skipping in endogenous HDAC6 mRNA and stably expressed HDAC6 reporter transcripts

Three of the HeLa HDAC6 clones that stably express HDAC6 minigene were transfected with Son siRNA to test splicing patterns of HDAC6 minigene. Son depletion in three of the HeLa HDAC6 stable cell lines (Clones 69, 57 and 65) resulted in skipping of exons 27 and 28 in both exogenous (Fig. 8C, 9C and 10C) and endogenous HDAC6 transcripts (Fig. 8D, 9D and 10D).

Figure 6: Primary Screening of Stable cell lines.

RNA extracted from G-418-resistant colonies was used to perform RT-PCR to amplify HDAC6 minigene transcripts. The bands migrating at 528 bp correspond to properly spliced reporter transcripts and the bands at 259 bp correspond to products in which exons 27 and 28 are skipped (A, B and C). RT-PCR was performed on RNA isolated from cells that were transiently transfected with the HDAC6 reporter plasmid (panel A: lanes 1 and 2; panel B: lane 1; panel C: lanes 1 and 2) as well to indicate the sizes of expected bands for comparison with bands from stable clones. Additional bands in some of the clones may be due to the presence of genomic DNA in the samples.

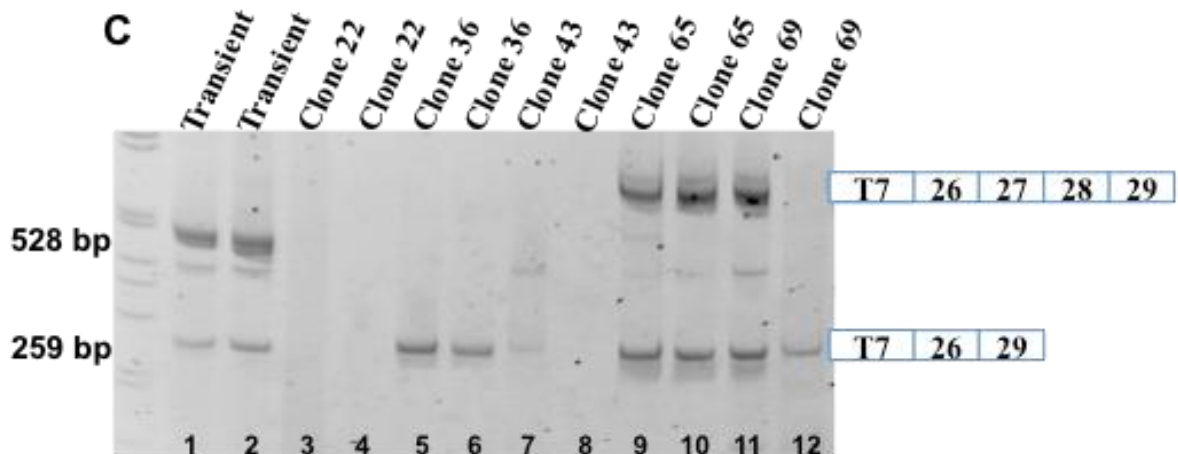
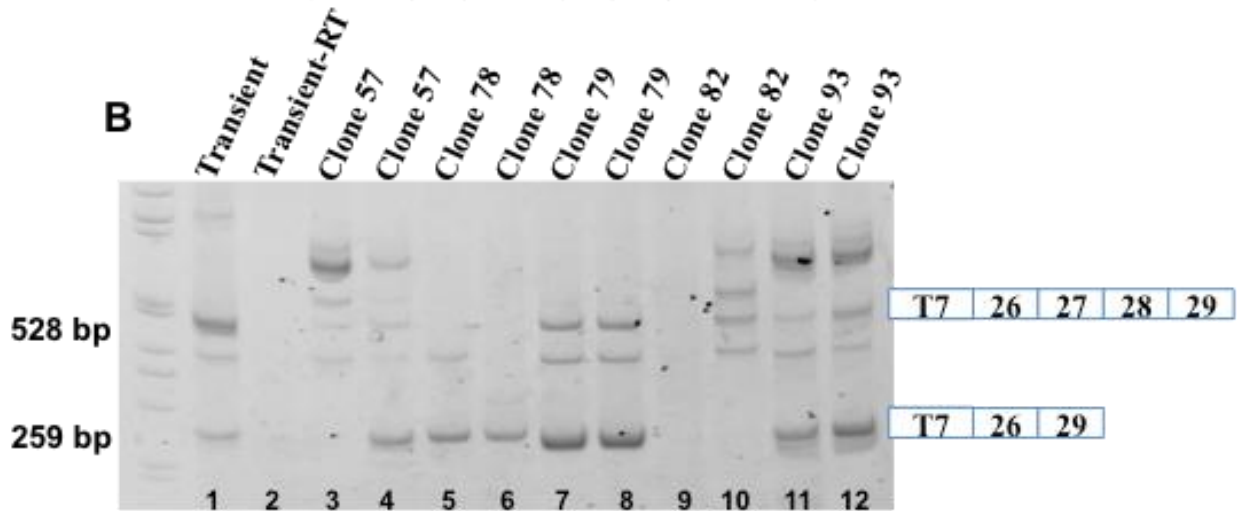
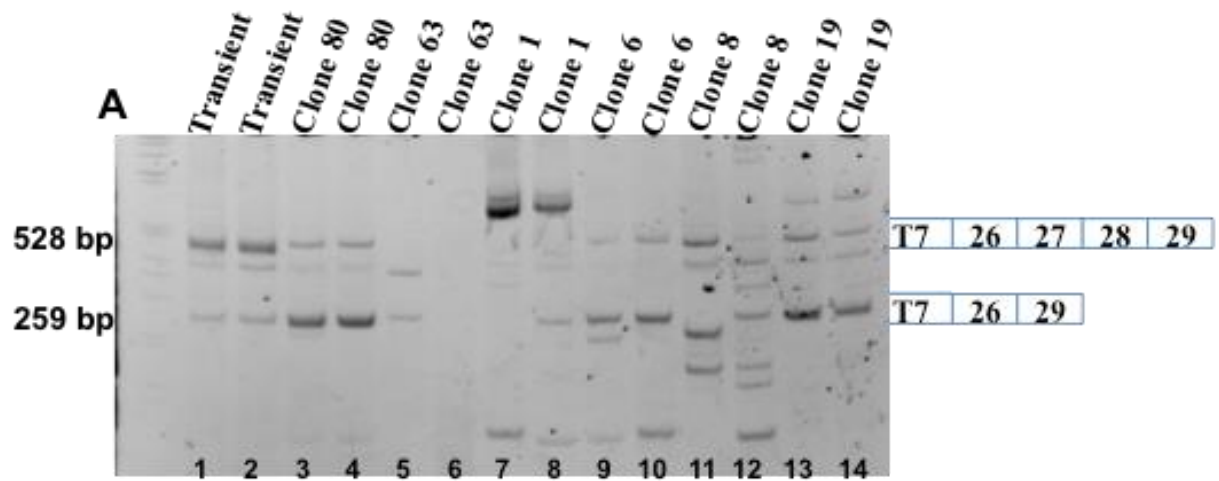


Figure 7: Secondary Screening of Stable cell lines.

Positive clones from the primary screening of stable reporter cell lines were expanded. RNA was extracted to perform RT-PCR to amplify HDAC6 minigene transcripts. The bands migrating at 528 bp correspond to properly spliced reporter transcripts and the bands at 259 bp correspond to products in which exons 27 and 28 are skipped (A, B and C). RT-PCR was performed on RNA isolated from cells that were transiently transfected with the HDAC6 reporter plasmid (panel A, B, C: lane 1) as well to indicate the sizes of expected bands for comparison with bands from stable clones. Additional bands in some of the clones may be due to the presence of genomic DNA in the samples.

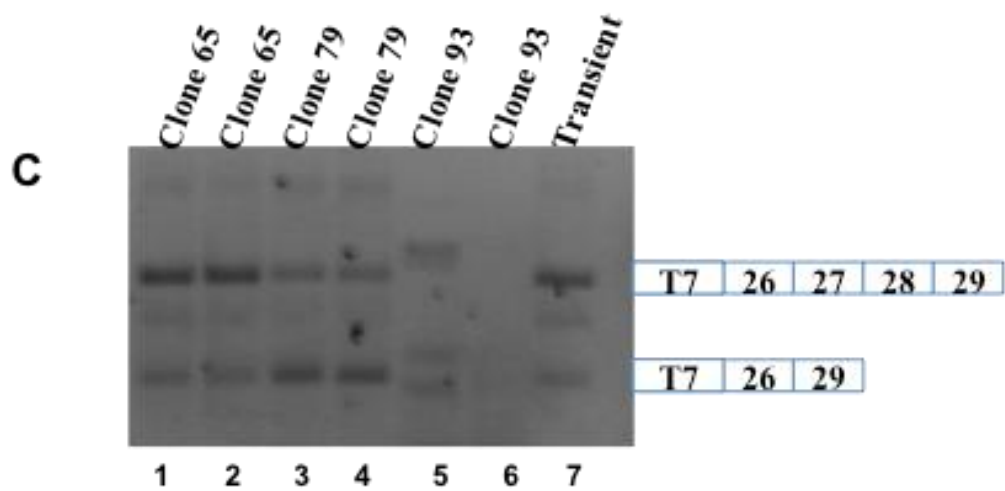
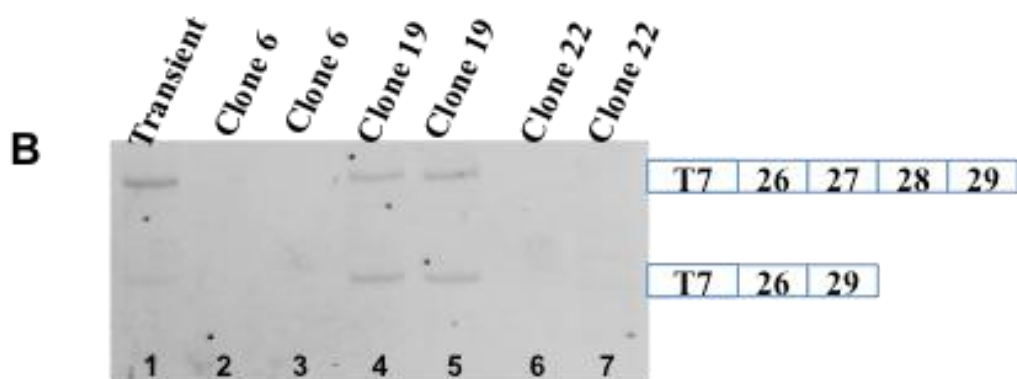
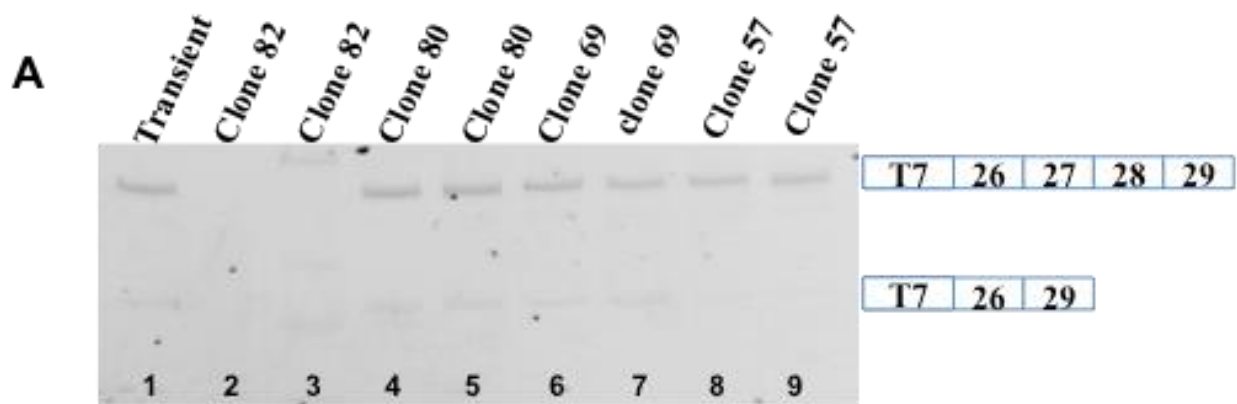
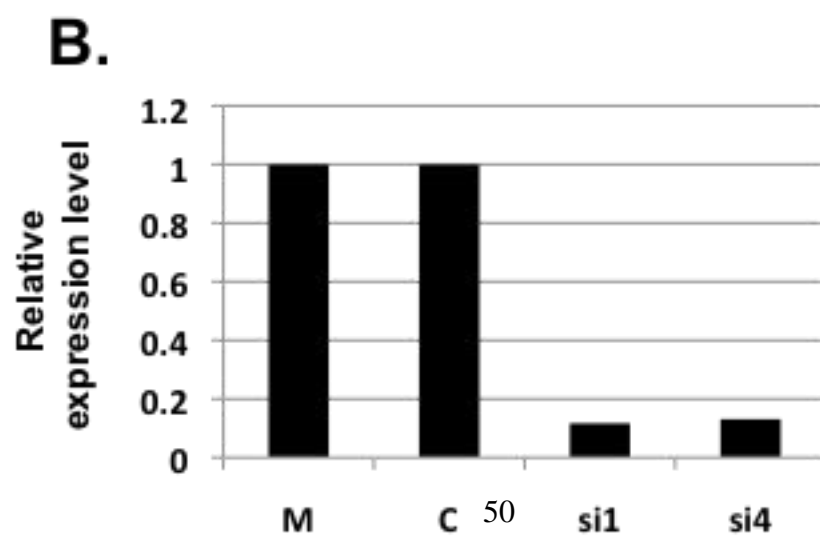
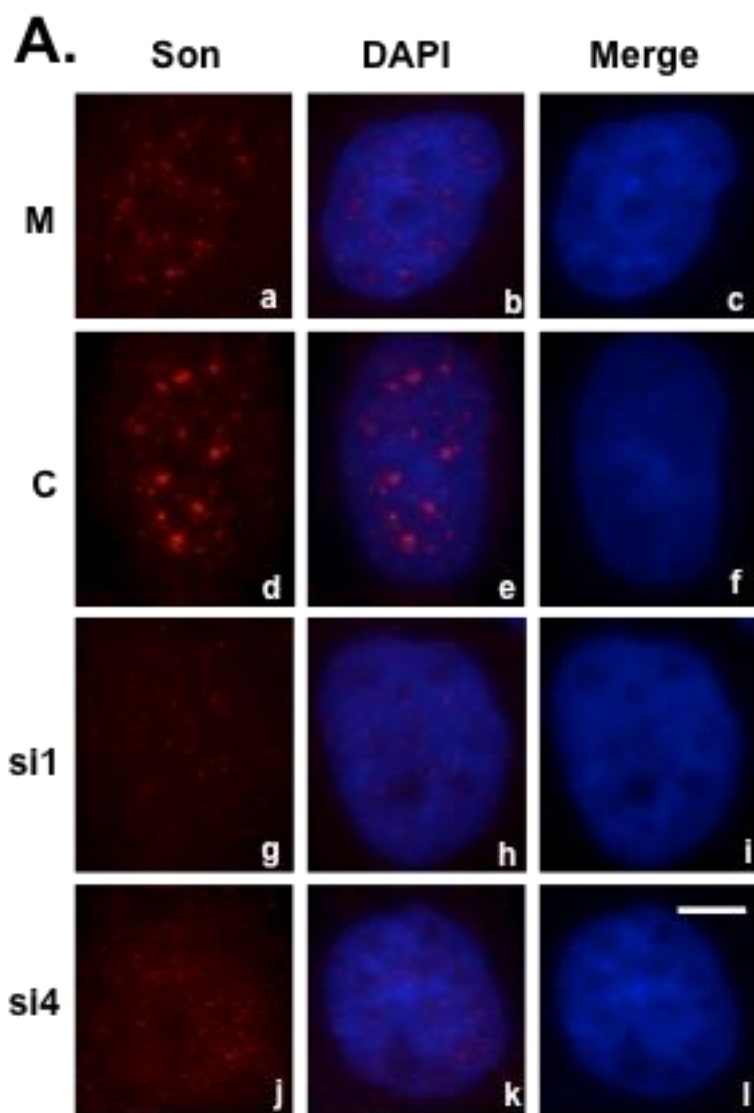
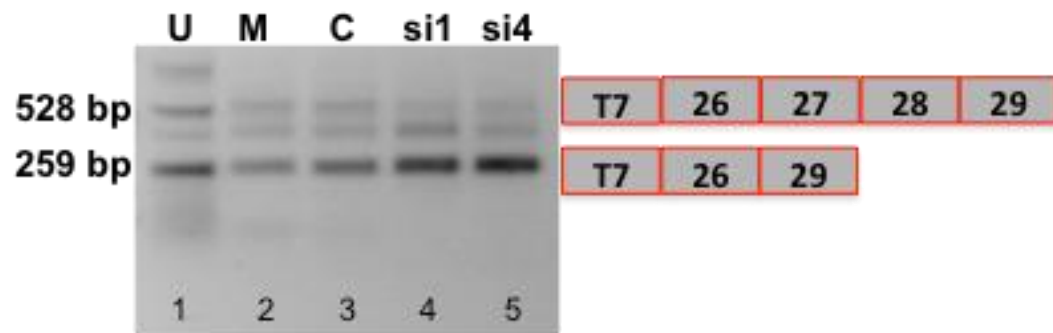


Figure 8: Son depletion causes exon skipping in HDAC6 reporter cell line 69.

HeLa HDAC6 clone 69 stably expressing HDAC6 reporter plasmid was transfected with Son siRNA duplexes and processed for either immunofluorescence (A) or harvested to perform qRT-PCR (B) to show Son depletion. Cells treated with Son siRNA and immunostained with anti-Son antibodies in (A) do not show Son labeling in nuclear speckles (g and j) when compared to mock and control siRNA treated cells (a and d). DNA was stained with DAPI (c, f, I and l). Bar = 5µm. qRT-PCR in (B) showed reduction of Son mRNA levels in siRNA-treated samples when compared to mock and control siRNA treated samples. (C) qRT-PCR performed in triplicate using primers that amplify only reporter minigene transcripts show exon skipping in Son siRNA-treated samples. (D) RT-PCR performed with primers that amplify both the endogenous as well as reporter minigene transcripts show exon skipping in siRNA-treated samples. Lanes labeled “U” represent the samples for which RNA was extracted without treating with siRNA reagents.



C.



D.

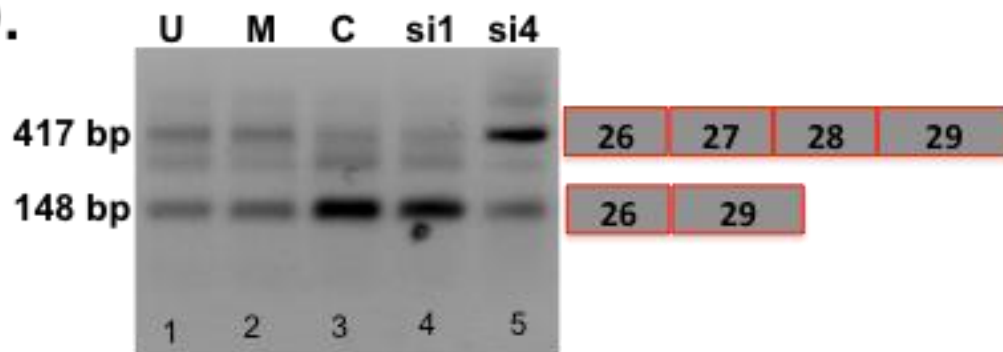
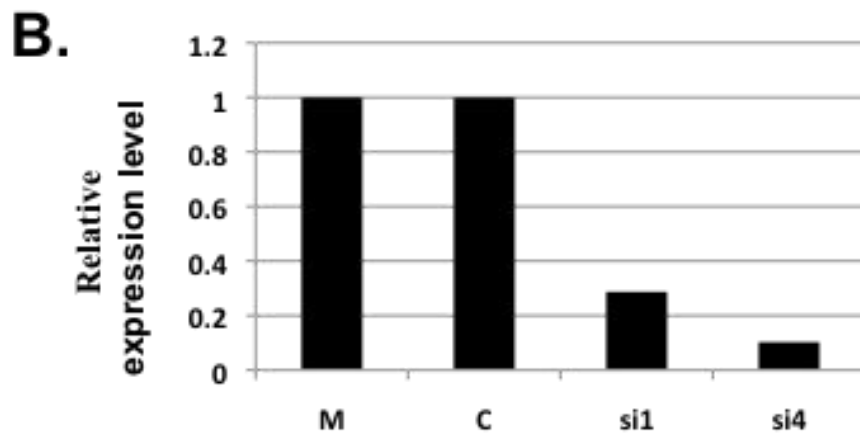
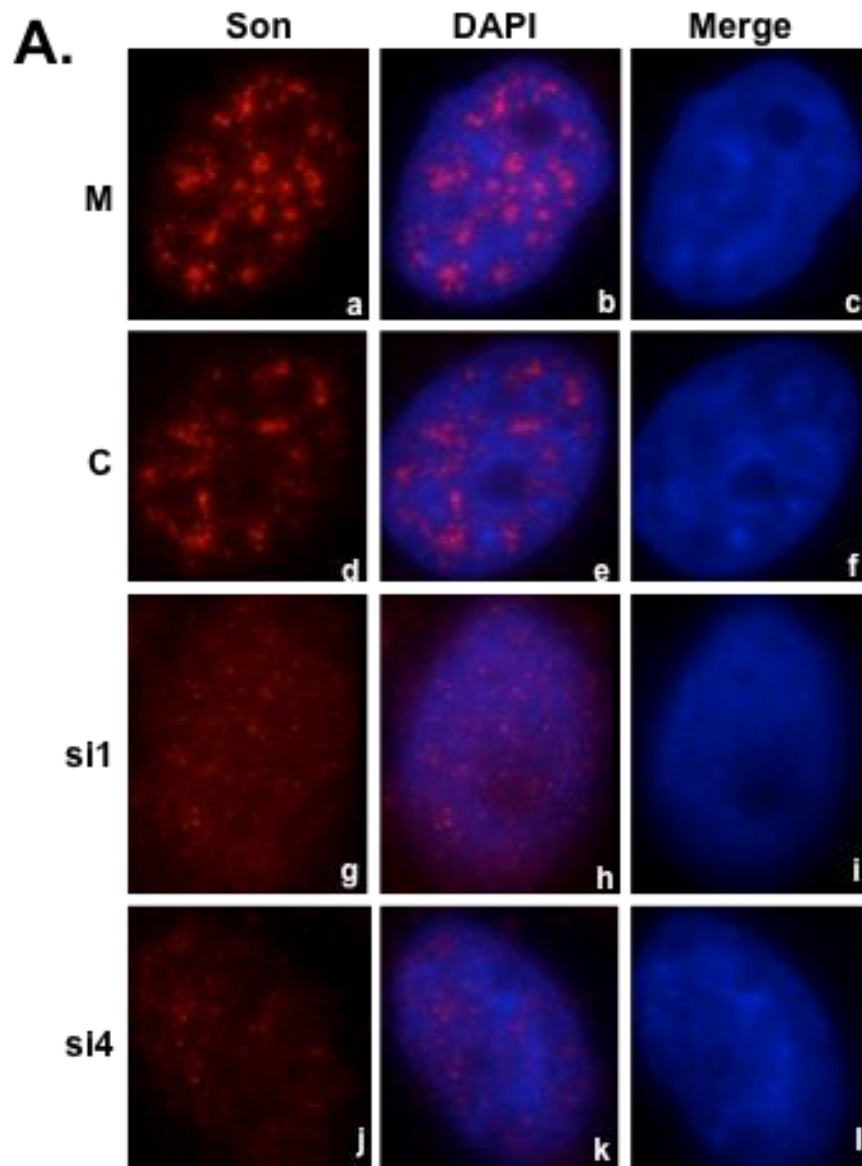
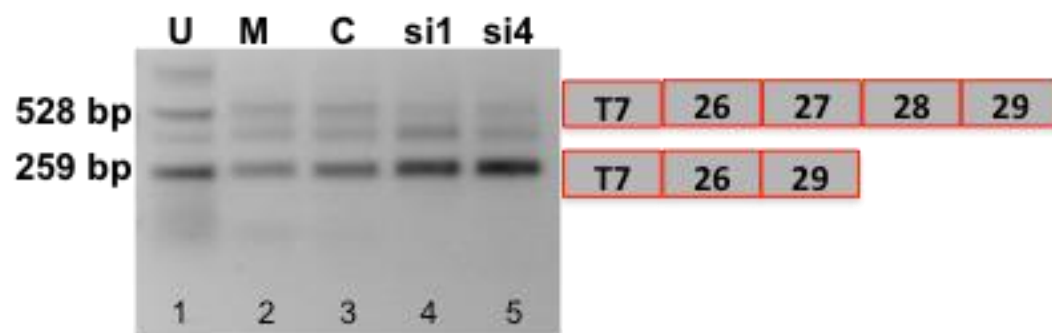


Figure 9: Son depletion causes exon skipping in HDAC6 reporter cell line 65.

HeLa HDAC6 clone 65 stably expressing HDAC6 reporter plasmid was transfected with Son siRNA duplexes and processed for either immunofluorescence (A) or harvested to perform qRT-PCR (B) to show Son depletion. Cells treated with Son siRNA and immunostained with anti-Son antibodies in (A) do not show Son labeling in nuclear speckles (g and j) when compared to mock and control siRNA treated cells (a and d). DNA was stained with DAPI (c, f, I and l). Bar = 5µm. qRT-PCR in (B) showed reduction of Son mRNA levels in siRNA-treated samples when compared to mock and control siRNA treated samples. (C) qRT-PCR performed in triplicate using primers that amplify only reporter minigene transcripts show exon skipping in Son siRNA-treated samples. (D) RT-PCR performed with primers that amplify both the endogenous as well as reporter transcripts show exon skipping in siRNA-treated samples. Lanes labeled “U” represent the samples for which RNA was extracted without treating with siRNA reagents.



C.



D.

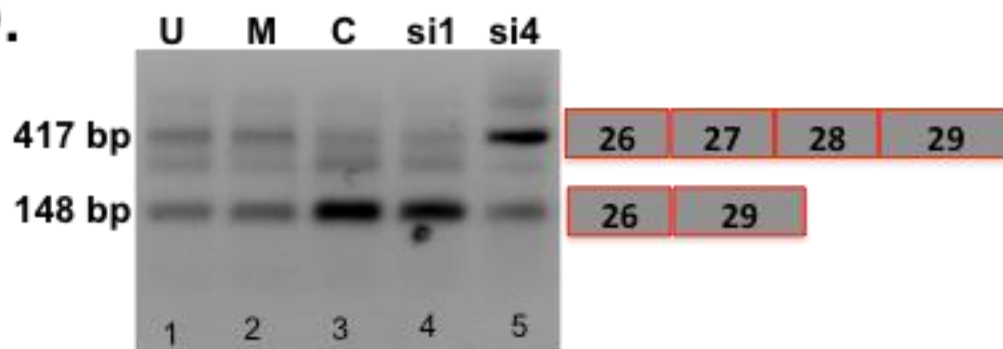
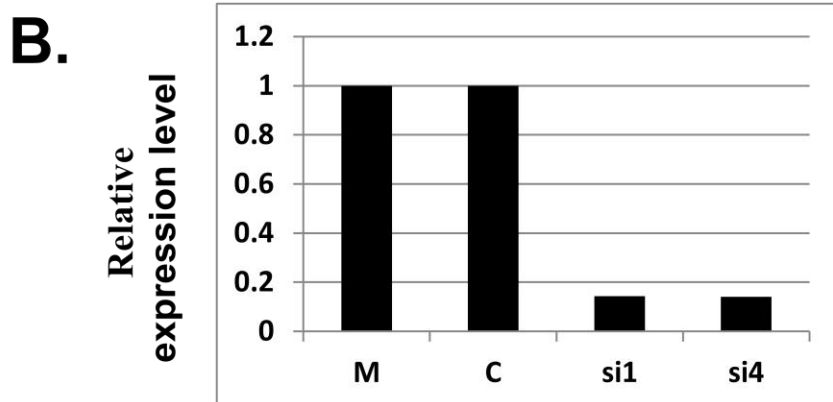
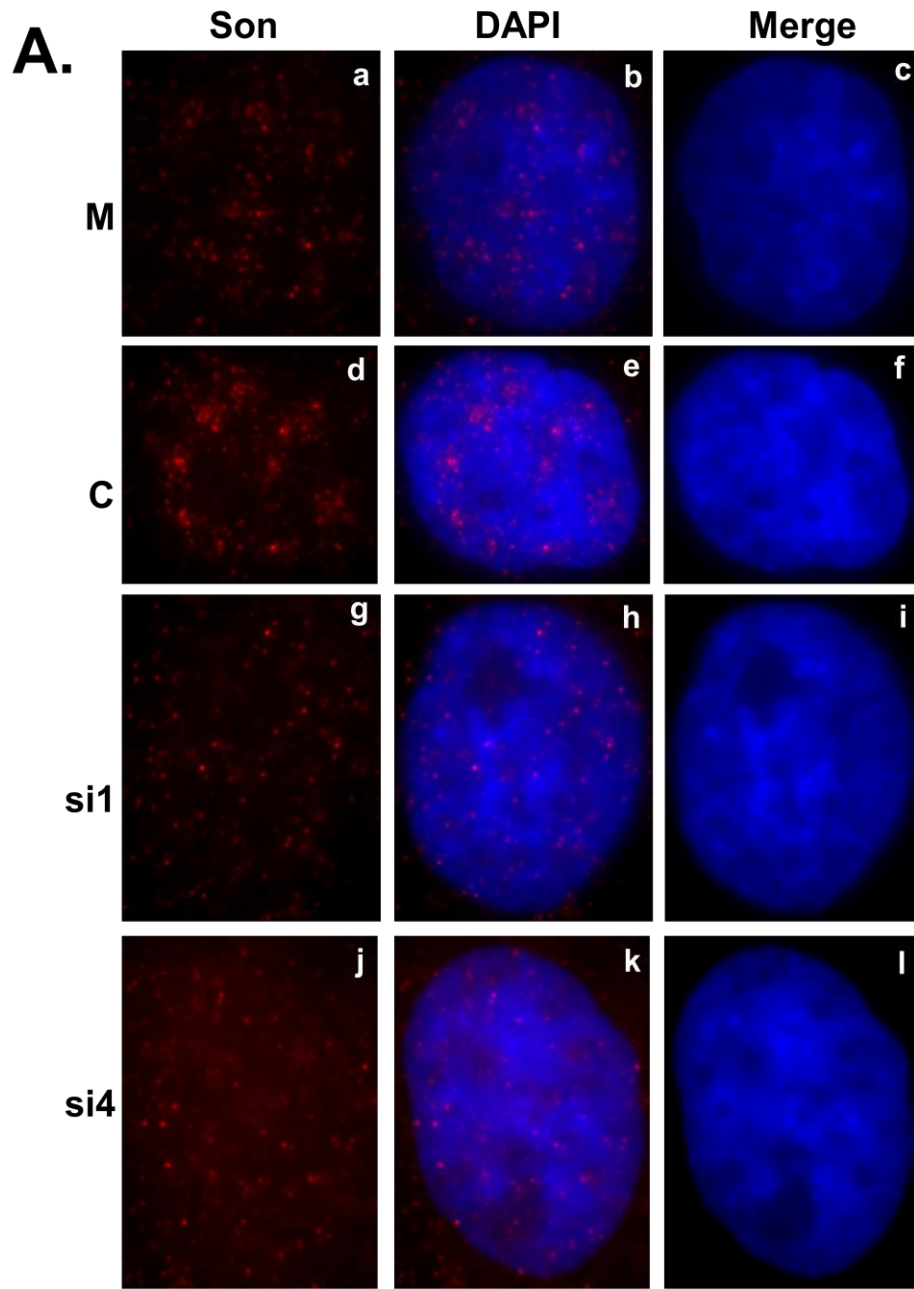
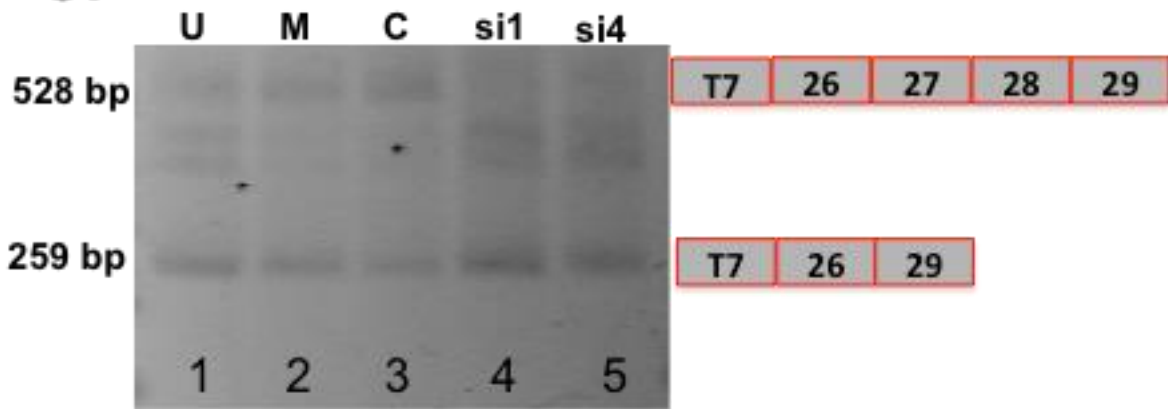


Figure 10: Son depletion causes exon skipping in HDAC6 reporter cell line 57

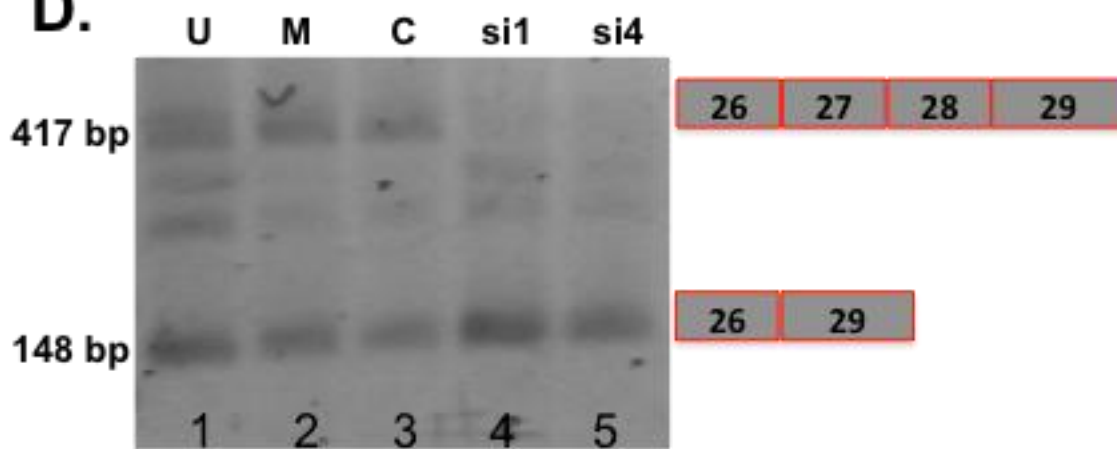
HeLa HDAC6 clone 57 stably expressing HDAC6 reporter plasmid was transfected with Son siRNA duplexes and processed for either immunofluorescence (A) or harvested to perform qRT-PCR (B) to show Son depletion. Cells treated with Son siRNA and immunostained with anti-Son antibodies in (A) do not show Son labeling in nuclear speckles (g and j) when compared to mock and control siRNA treated cells (a and d). DNA was stained with DAPI (c, f, I and l). Bar = 5µm. qRT-PCR in (B) showed reduction of Son mRNA levels in siRNA-treated samples when compared to mock and control siRNA treated samples. (C) qRT-PCR performed in triplicate using primers that amplify only reporter minigene transcripts show exon skipping in Son siRNA-treated samples. (D) RT-PCR performed with primers that amplify both the endogenous as well as reporter transcripts show exon skipping in siRNA-treated samples. Lanes labeled “U” represent the samples for which RNA was extracted without treating with siRNA reagents.



C.



D.



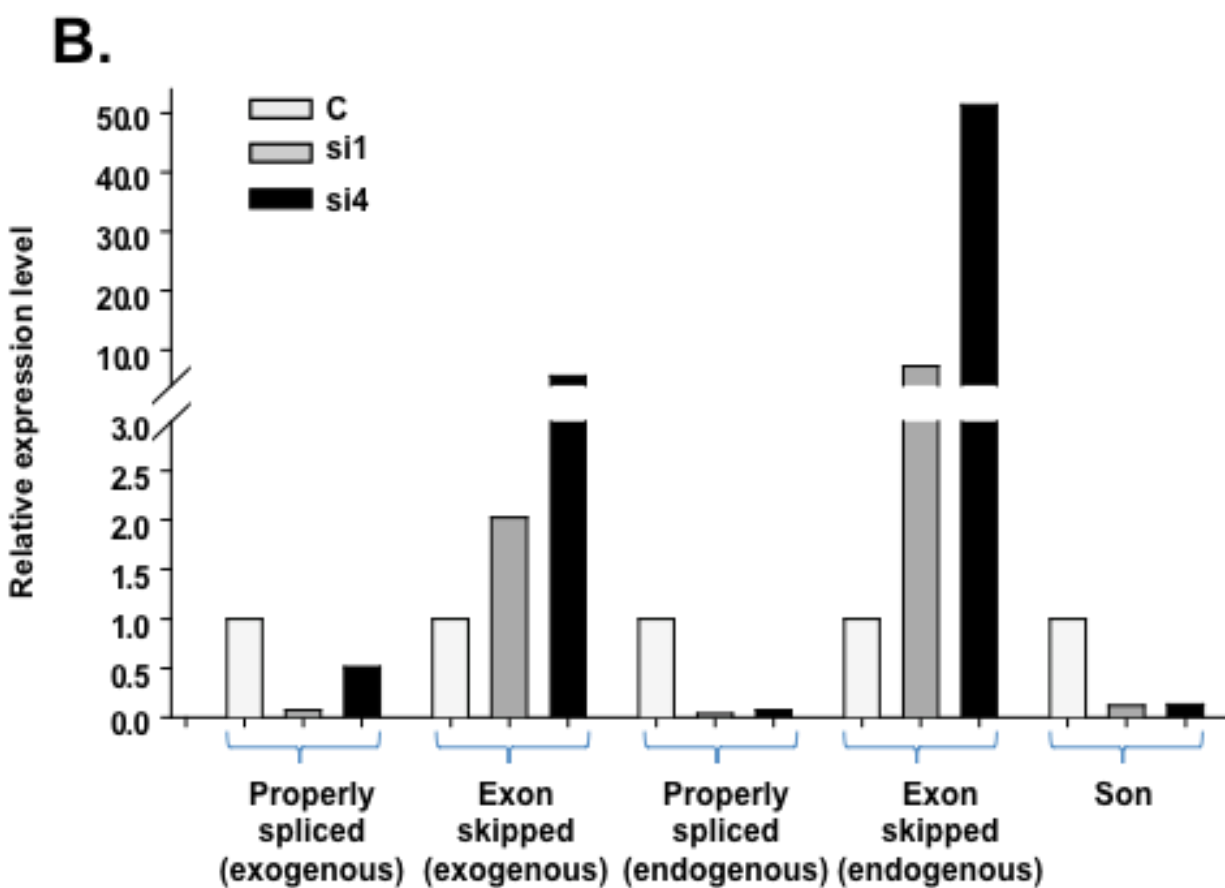
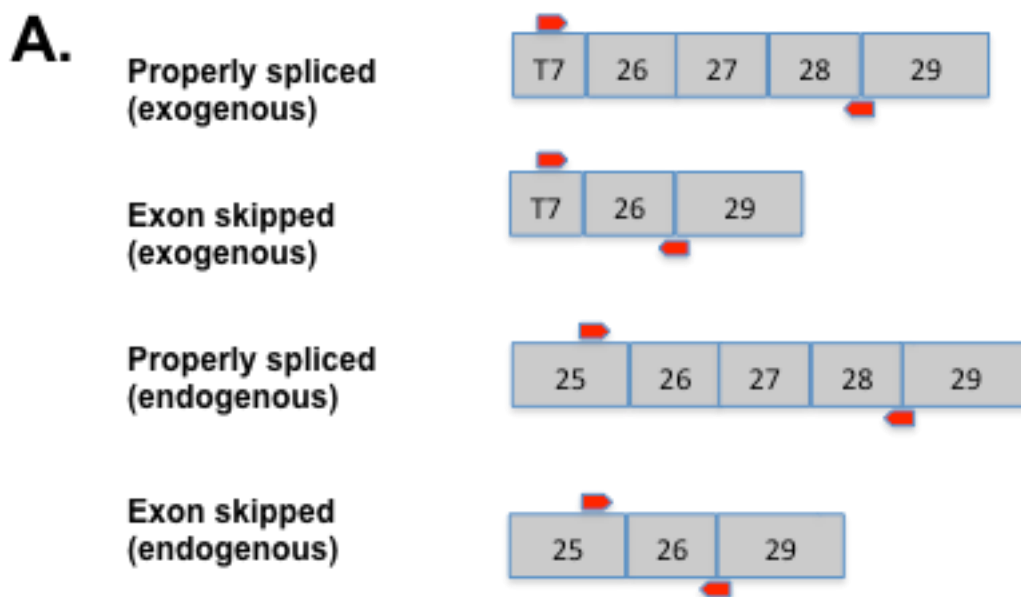
Exon skipping of HDAC6 reporter transcripts in Son depleted cells when expressed transiently or stably indicates that the HDAC6 minigene reporter construct contains the necessary elements required to study Son-dependent splicing of exons 27 and 28. Since multiple stable cell lines expressing the HDAC6 reporter minigene show the same result, there is probably no connection between chromatin context and splicing regulation by Son for splicing of exons 27 and 28 of HDAC6 transcripts.

4.5 qRT-PCR analysis of HDAC6 exon skipping in Son-depleted cells

To quantify the relative expression level of properly spliced and exon skipped products in Son depleted cells that stably express HDAC6 reporter minigene, exon-junction primers were designed that allow the amplification of single products as shown in fig. 11A. Properly spliced HDAC6 minigene contains all exons i.e., exons 26, 27, 28 and 29 allowing us to design an exon junction primer that includes the last 12 nt of exon 26 and first 12 nt of exon 27. Since exon 27 is absent in the exon skipped product, the EJ26/27 primer specifically complements the properly spliced HDAC 6 transcripts. Likewise, exons 26 and 29 are joined when exon skipping occurs. An exon junction primer (EJ26/29) was designed containing the last 12 nt of exon 26 and the first 12 nt of exon 29, it will amplify only the reporter transcripts in which exons 27 and 28 are skipped. Furthermore, to differentiate between endogenous and exogenous HDAC6 transcripts, a primer was designed to complement sequences within exon 25 to amplify only endogenous HDAC6 transcripts (since HDAC6 minigene does not contain exon 25), and another primer was designed to complement sequences just following the T7 promoter region of the HDAC6 minigene to amplify only exogenous HDAC6 transcripts (since the T7 sequences are absent in

Figure 11: qRT-PCR analysis of HDAC6 exon skipping in Son-depleted cells.

qRT-PCR performed on HeLa HDAC6 stable cells post-Son depletion. A) Four sets of exon junction primers were used to amplify splice variants for exogenous reporter transcripts as well as for endogenous HDAC6 transcripts. B) Relative transcript level for properly spliced versus exon skipped transcripts is measured in both endogenous and exogenous HDAC6.



endogenous HDAC6 transcripts). qRT-PCR was performed in the Son-depleted HeLa HDAC6 stable cell line (clone 69) using combination of primers for HDAC6. Son depletion was validated by qRT-PCR. For both the endogenous and the exogenous HDAC6 transcripts in Son-depleted cells, the level of properly spliced transcripts were low while levels of improperly spliced transcripts were high in comparison to respective transcript levels in cells treated with luciferase siRNA (control) (Fig. 11B). This indicates the efficacy of the minigene reporter system we generated in mimicking the endogenous HDAC6 splicing patterns.

4.6 HeLa HDAC6 stable cell line with single reporter transcription locus

The stable cell lines constructed using HDAC6 reporter minigene construct were used to observe reporter transcription loci inside the cells. To achieve this, RNA fluorescence *in situ* hybridization (RNA-FISH) was initially performed for all six positive clones.

4.6.1 Screening HeLa HDAC6 stable cell lines to identify reporter transcription locus

Two RNA-FISH probes were designed one at the T7 promoter region which binds only to reporter minigene transcripts and the other probe in exon 26 region of HDAC6 which can hybridize to both exogenous and endogenous HDAC6 transcripts. The stable clones were grown on coverslips and RNA-FISH combined with immunofluorescence was performed as described in the methods section. For immunofluorescence, antibodies against Son and SF2/ASF were used to observe co-localization patterns to HDAC6 transcripts. Since BTM cell line constructed by Dr. Paula Bubulya consists of the same plasmid backbone and

includes the T7 promoter region, RNA-FISH was performed on this cell line as a control. The BTM cell line is considered a positive control for HDAC6 reporter since it can be used to validate the working conditions of FISH probes and other reagents used. RNA-FISH in HeLa cells was performed as a negative control that will not contain T7 sequences and can be used to establish background fluorescence levels of RNA-FISH probes.

Unfortunately, RNA-FISH performed on stable cell HDAC6 reporter lines showed no consistent labeling of transcription loci. BTM cell line hybridized with T7 probe showed a bright transcription locus (Fig. 12: panel 1) and co-localization with Son (Fig.12 panel 2) and SF2/ASF (Fig. 12 panel 3) indicating that the probes and other reagents used were working appropriately. HeLa cells in the absence of probe did not show any transcription loci (Fig. 12 panels 9, 10 and 11) and HeLa cells hybridized with E26 probe show random labeling of bright dots, some of which co-localized with Son and SF2/ASF (Fig. 12 panels 5, 6 and 7). HDAC6 stable cell lines hybridized with E26 probe showed either 2-3 bright dots that resembled a nuclear speckle pattern. The speckle pattern observed colocalizes with Son and SF2/ASF while some of the bright dots colocalize with Son and SF2/ASF (Fig.12 panels a-x).

4.6.2 Screening HeLa HDAC6 stable populations to identify reporter transcription locus

Since we did not successfully generate a stable clone with single transcription locus, we proceeded to make stable populations with HDAC6 reporter minigene construct and visualize the transcription loci of HDAC6 minigene *in situ*. HeLa cells were transfected with HDAC6 reporter construct and selected using G418 (1 mg/ml). The cells were then trypsinized, collected and frozen stocks were made. RNA-FISH with different probe concentrations (10

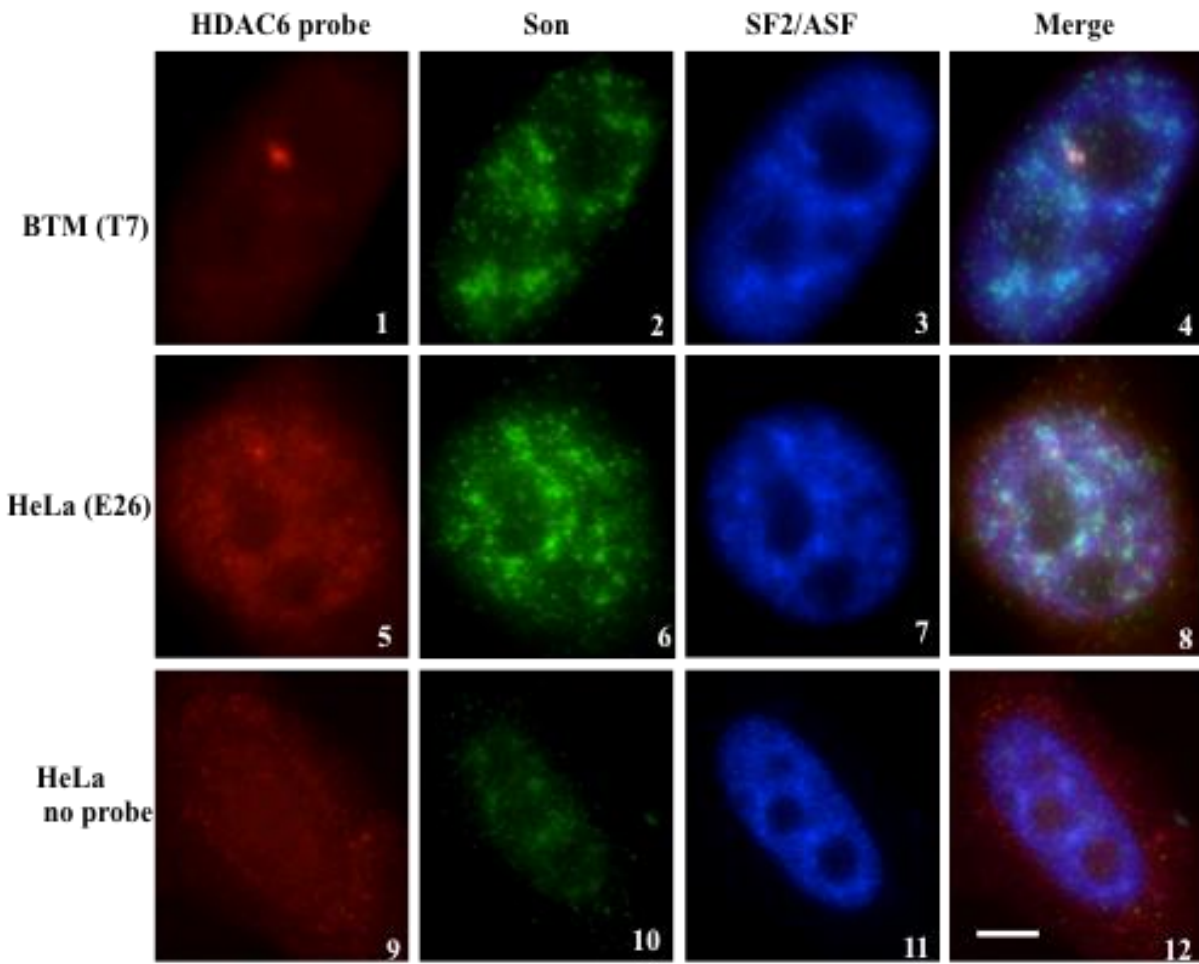
ng, 20 ng and 40 ng), combined with immunofluorescence with antibodies against Son and SF2/ASF was performed. RNA-FISH with HDAC6 probe showed speckle like structures (Fig.13 panels a, e and i) that co-localized with Son and SF2.ASF (Fig.13 panels b, c, d, f, g, h, j, k and l).

4.6.3 Hybridization of transiently expressed HDAC6 reporter minigene to identify transcription/splicing factories

RNA-FISH for both stable populations and stable cell lines being unsuccessful, we moved on to cells transiently expressing HDAC6 reporter minigene. Transient transfection of minigenes such as BTM have previously been used to create large transcription factories within nuclei that can be detected by RNA-FISH (Huang and Spector, 1996). HeLa cells transfected with 2 µg of HDAC6 reporter plasmid DNA were fixed after 48 hours to perform RNA-FISH with different probe concentrations (10 ng, 20 ng and 40 ng) and Immunofluorescence with antibodies against Son and SF2/ASF. RNA-FISH with E26 probe showed 2-3 bright dots (Fig.14 panels a, e and i) some of them colocalizing with Son and SF2/ASF (Fig.14 panels b, c, d, f, g, h, j, k and l). One more condition was tested by varying the amount of DNA used for transfection. HeLa cells were transfected with different concentrations of HDAC6 reporter plasmid DNA (1, 2, 5, 7 and 10 µg), and RNA-FISH combined with immunofluorescence was performed after two days. Again, transcription factories were not observed, but we observed a nuclear speckle pattern for HDAC6 reporter transcripts (Fig.15 panels a, e, i, m and q).

Figure 12: Screening HeLa HDAC6 stable cell lines to visualize the reporter transcription locus by RNA-FISH.

HeLa HDAC6 stable cell lines were processed for RNA-FISH to label HDAC6 minigene transcripts combined with dual label immunofluorescence localization of Son and SF2/ASF. Panels 1, 5, a, e, i, m, q and u show RNA-FISH signal in different cell lines as labeled. Panel 9 shows HeLa cells not labeled with any probe (to determine background fluorescence). Panels 2, 6, 10, b, f, j, n, r and v show localization of Son in different cell lines. Panels 3, 7, 11, c, g, k, o, s and w show localization of SF2/ASF in different cell lines. Bar = 5 μ m.



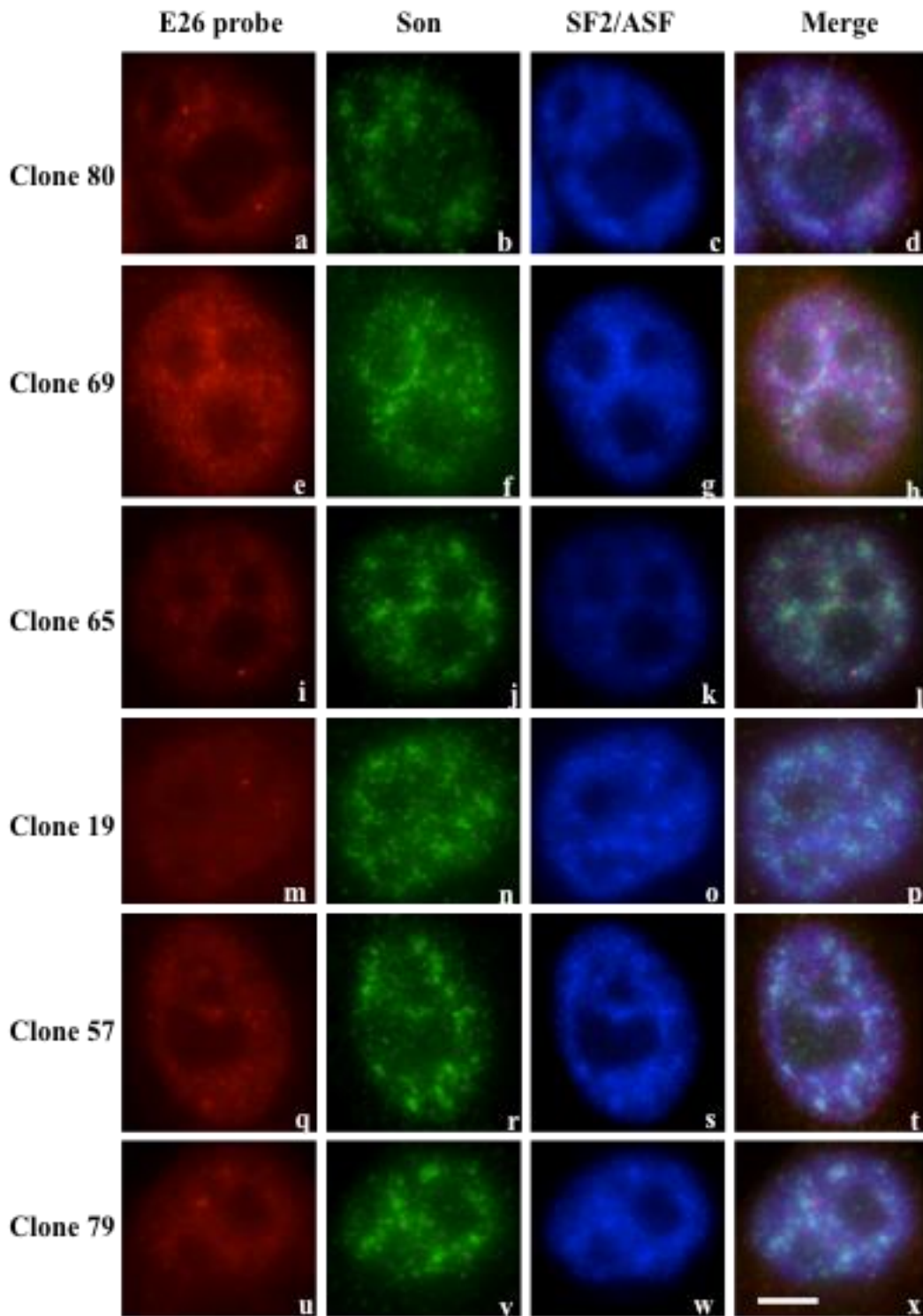


Figure 13: Screening HeLa HDAC6 stable populations by RNA-FISH to identify reporter transcription locus.

HeLa HDAC6 stable populations were processed for RNA-FISH to label HDAC6 minigene transcripts combined with dual label immunofluorescence localization of Son and SF2/ASF. Panels a, e and i show HDAC6 minigene transcripts of stable populations when 10, 20 or 40 ng of E26 probe was used for RNA-FISH, respectively. Panels b, f and j show localization of Son in stable populations. Panels c, g and k show localization of SF2/ASF in stable populations. Bar = 5 μ m.

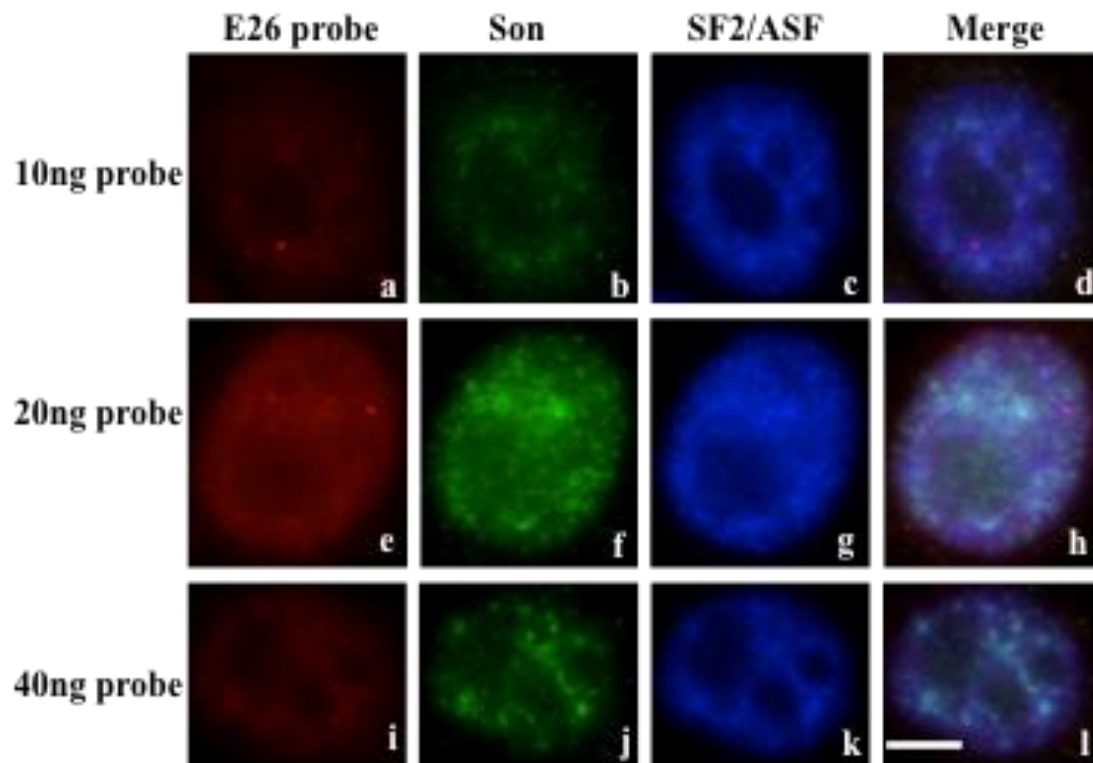


Figure 14: RNA-FISH of transiently expressed HDAC6 reporter minigene transcripts to visualize transcription/splicing factories.

HeLa cells were transiently transfected with 2 µg of HDAC6 minigene reporter plasmid. After 48 hours, the cells were fixed and processed for RNA-FISH to label HDAC6 minigene transcripts combined with dual-label immunofluorescence to visualize localization of Son and SF2/ASF.

Panels a, e and i show transiently expressed HDAC6 minigene transcripts when 10, 20 and 40 ng of E26 probe was used for RNA-FISH, respectively. Panels b, f and j show localization of Son.

Panels c, g and k show localization of SF2/ASF. Bar = 5µm.

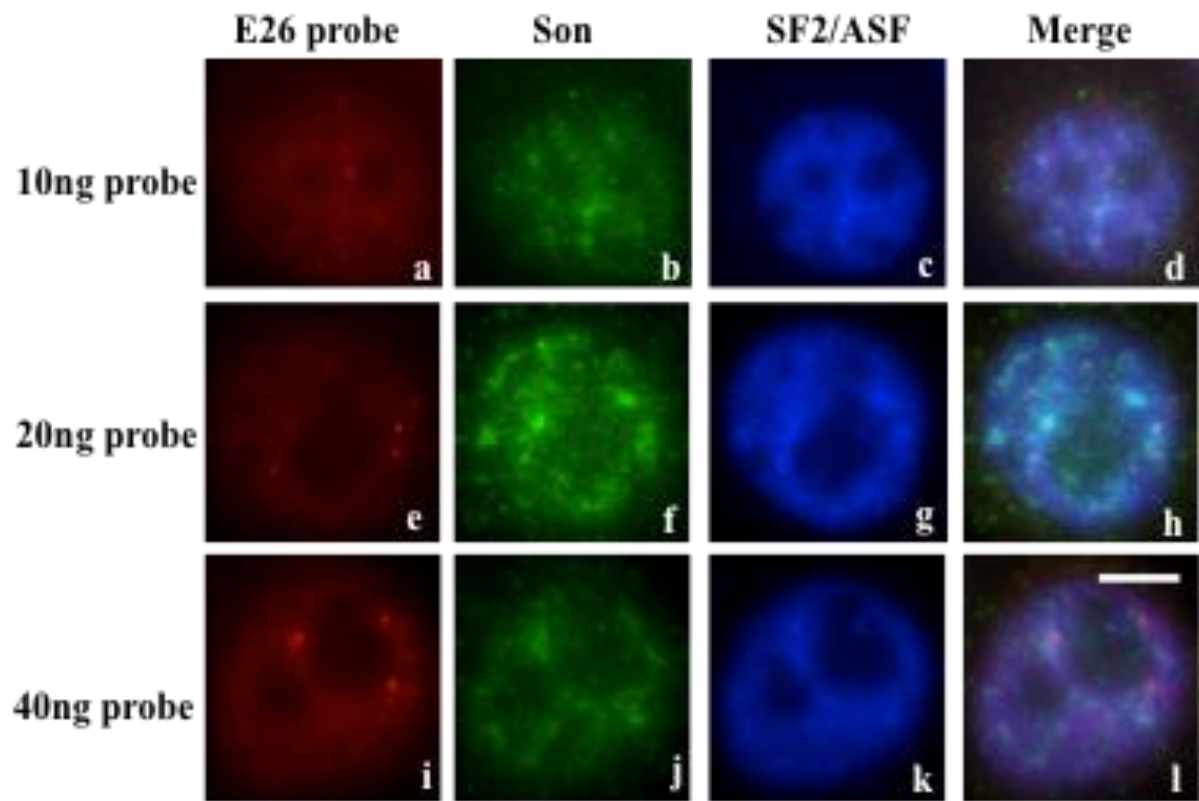
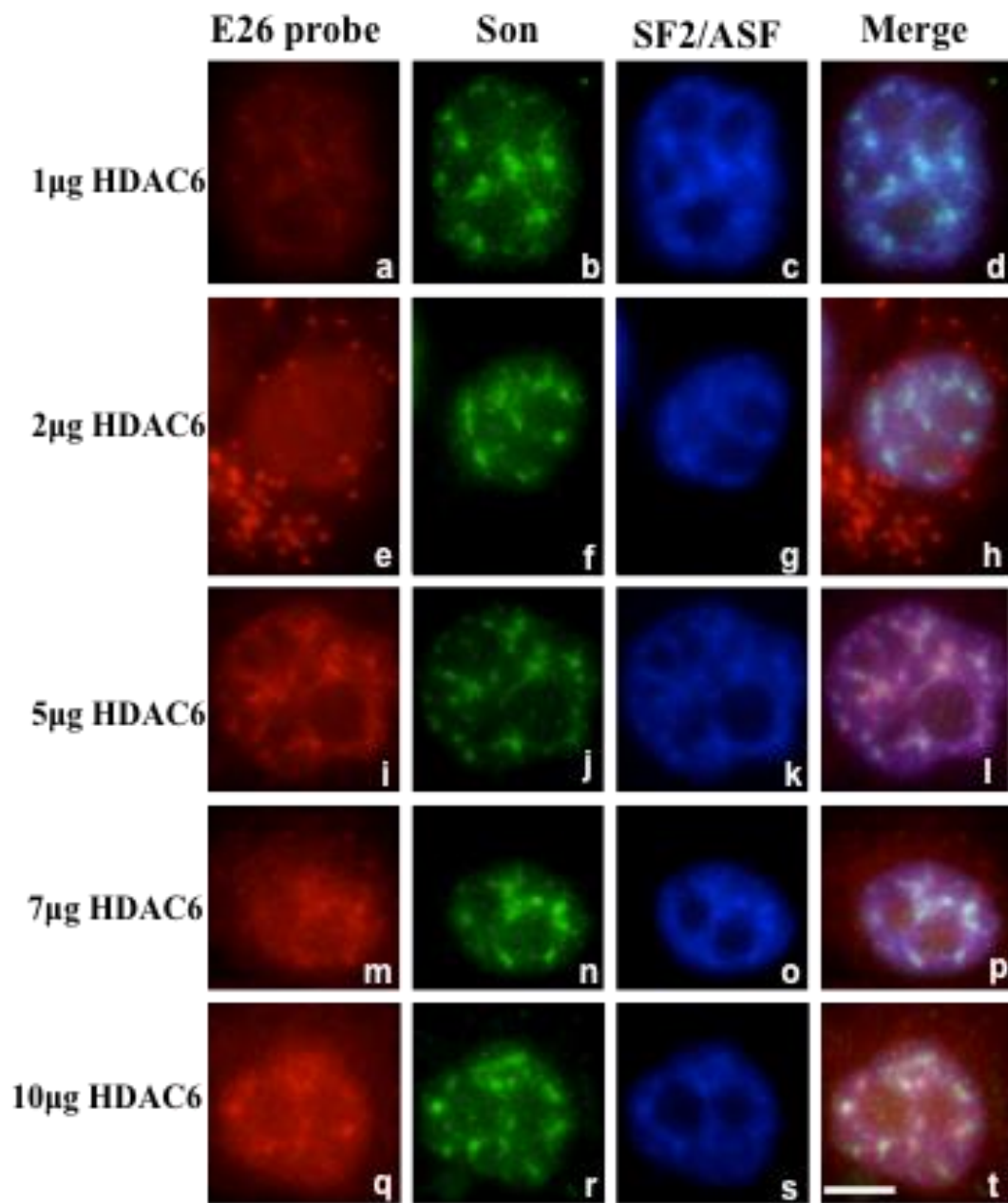


Figure 15: RNA-FISH of transiently expressed HDAC6 reporter minigene transcripts to visualize transcription/splicing factories.

HeLa cells were transiently transfected with various amounts (1, 2, 5, 7 and 10 μg) of HDAC6 minigene reporter plasmid. After 48 hours, the cells were fixed and processed for RNA-FISH to label HDAC6 minigene transcripts combined with dual label immunofluorescence to visualize localization of Son and SF2/ASF. Panels a, e and i show transiently expressed HDAC6 minigene RNA when either 1, 2, 5, 7 or 10 μg of HDAC6 minigene reporter plasmid was transfected, respectively. Panels b, f and j show localization of Son. Panels c, g and k show localization of SF2/ASF. Bar = 5 μm .



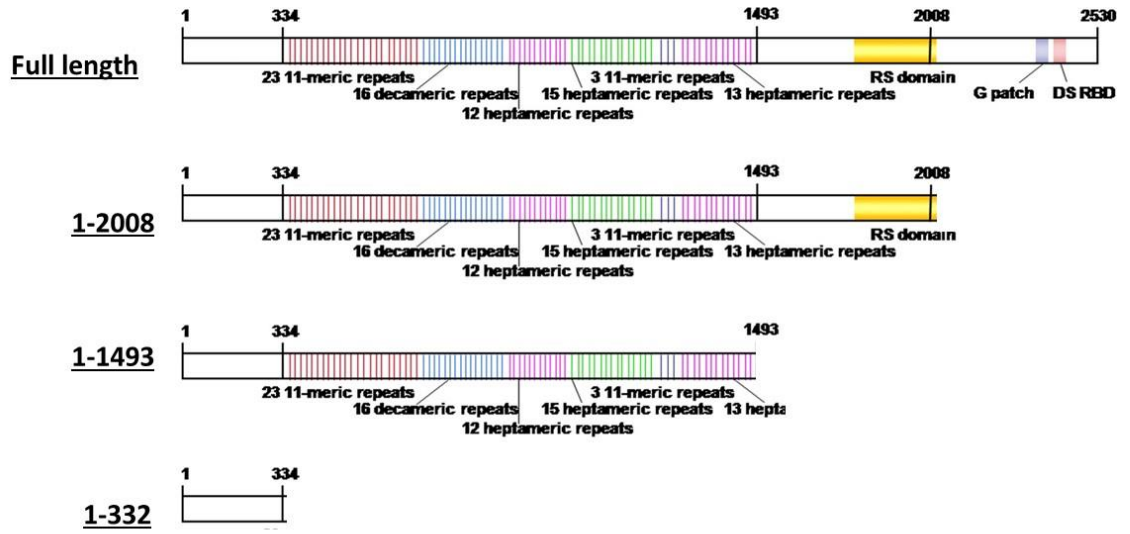
4.7 Stable cell lines constructed in HEK 293 with YFP tagged Son deletion mutant constructs express YFP-Son constantly

HEK 293 cells were transfected with four YFP tagged siR resistant Son deletion mutant constructs namely YFP-siR-Son Fl, YFP-siR-Son 1-2008, YFP-siR-Son 1-1493 and YFP-siR-Son 1-332 (see Figure 16A). Stable clones were selected using G418. Primary and secondary screening through fluorescent microscopy showed consistent expression of the constructs. Three positive clones were picked for each construct after secondary screening. Clones 5, 27 and 64 (YFP-siR-Son Fl), 4, 32 and 69 (YFP-siR-Son 1-2008), 15, 48 and 58 (YFP-siR-Son 1-1493), 8, 16 and 46 (YFP-siR-Son 1-332) were selected (Figure 16B).

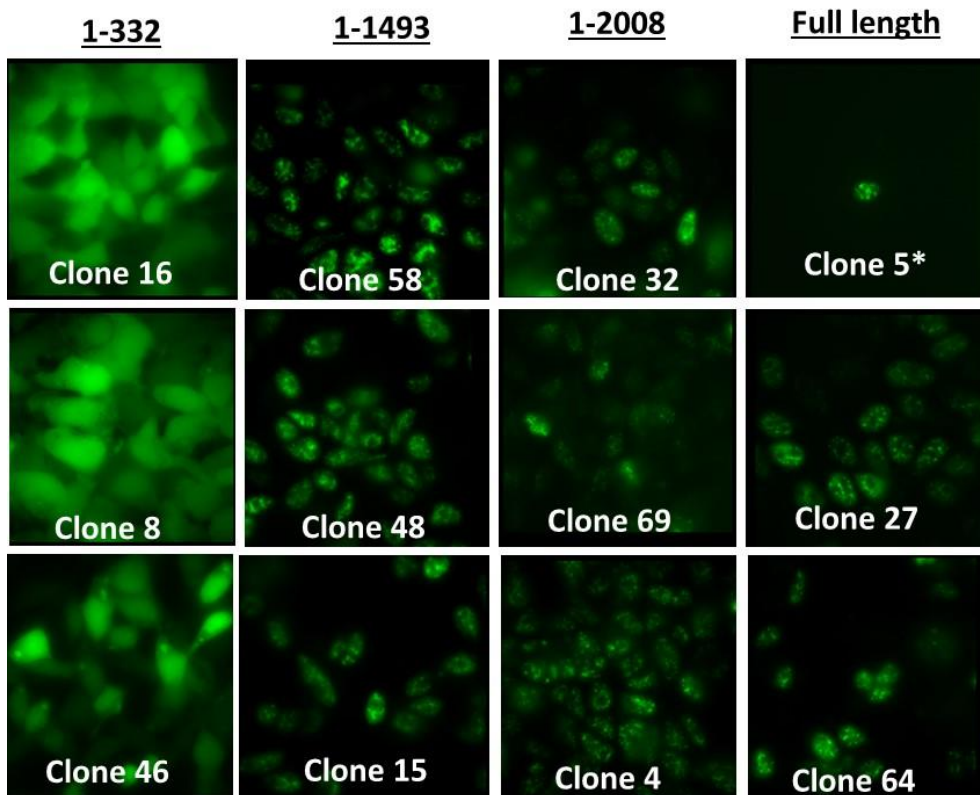
Figure 16: siRNA resistant Son Deletion Mutant constructs and their expression in Stable HEK 293 Cell lines.

- A. SiRNA resistant Son full-length mutant construct consists of all the domains of Son and in tagged to YFP. siRNA resistant Son 1-2008 deletion mutant construct consists of the region from 1 to 2008 amino acids and lacks C-terminal G-patch and double stranded DNA binding domain. siRNA resistant Son 1-1493 deletion mutant construct consists of the region from 1 to 1493 amino acids and lacks C-terminal RS domain, G-patch and double stranded DNA binding domain. siRNA resistant Son 1-332 deletion mutant construct consists of the region from 1 to 332 amino acids and lacks the repeat motifs and C-terminal RS domain, G-patch and double stranded DNA binding domain.
- B. Stable cell lines constructed using the four deletion mutant constructs were screened for consistent overexpression. The stable clones 5, 27 and 64 of full length-siR Son show speckled pattern confirming the overexpression of Son protein. The stable clones 4, 32 and 69 of 1-2008-siR Son show speckled pattern confirming the overexpression of Son protein. The stable clones 15, 48 and 58 of 1-1493-siR Son show speckled pattern confirming the overexpression of Son protein. The stable clones 8, 16 and 46 of 1-332-siR Son show expression of protein in both nucleus and cytoplasm confirming the loss of speckled pattern of overexpressed Son deletion mutant.

A



B



CHAPTER 5: DISCUSSION

Son is the largest SR protein identified in proteomic analysis of nuclear speckles (Saitoh et al., 2004). Our lab has previously showed that depletion of Son causes reorganization of nuclear speckles in to a doughnut shaped phenotype (Sharma et al., 2010). In the process of understanding the cellular functions of Son, microarray and exon array analysis performed in Son depleted cells showed decrease or increase in transcript levels as well as altered splicing of transcripts that encode proteins important for cellular processes such as cell cycle. Our lab has previously validated exon skipping in transcripts for three endogenous chromatin-regulating genes including HDAC6, ADA and SETD8 that is caused when Son is depleted (Sharma et al., 2011). Ahn et al (2011) discussed the effect of Son depletion on splicing of cell cycle regulatory proteins such as TUBG1, KATNB1, TUBGCP2, PCNT, AKT1 and AURKB.

Understanding the mechanism by which Son regulates splicing events of several selected genes requires a set of experiments to help define *cis*-acting elements (ESEs/ISEs) in transcripts that interact with Son for accurate splicing, as well as the domains of Son that mediate splicing activities, I aimed to determine the domains/repeats important for Son dependent splicing in HDAC6. Since Son is known to alter splicing of transcripts with weak splice sites (Sharma et al., 2011 and Ahn et al., 2011), we analyzed the splice sites of HDAC6 between exons 26 and 29 through ESEfinder. ESEfinder predicted weak splice sites at 5' splice site of exon 27 and 3' splice site of exon 28 that possibly explains why HDAC6 splicing is dependent on presence of Son. Further experiments need to be done to validate the presence of weak splice sites in HDAC6 and Son dependency for accurate splicing. This can be performed by introducing silent mutations in the predicted weak splice sites of HDAC6 and determining Son dependency for

accurate splicing. Moreover, Son depletion causes exclusion of exons 27 and 28 in HDAC6 transcripts that code for the BUZ domain of HDAC6 protein. Since the transcripts without exons 27 and 28 are still in frame at the junction of exons 26 and 29, we predict that the mRNA is translated and the protein produced lacks BUZ domain. There is no evidence of natural occurrence of HDAC transcripts lacking exons 27-28 in the literature. However, HDAC6 protein lacking the BUZ domain is predicted to be non-functional in binding to ubiquitin, thus interfering with the process of recruiting mis-folded proteins to aggresomes. Aggresomes are formed as a response to formation of misfolded proteins. Failure to degrade misfolded proteins results in neuronal cell death in many neurodegenerative diseases. Polyubiquitinated misfolded proteins are transported to aggresomes by dynein motor proteins. HDAC6 binds to dynein motor proteins via a dynein-motor binding (DMB) domain present in between the two deacetylase domains, and it binds to polyubiquitinated misfolded proteins through its BUZ domain. Thus HDAC6 helps recruit misfolded proteins to dynein motor proteins followed by their transport to aggresomes (Kawaguchi et al., 2003; Rodriguez-Gonzalez et al., 2008).

To gain insight into splicing functions for Son, we relied on reporter minigene assays. Our goal was to construct a reporter minigene system inside cells that could be used for RNA-FISH combined with immunofluorescence with antibodies against Son, several other splicing factors and splicing repressors such as hnRNPs in order to determine the effects of Son depletion on co-recruitment of splicing regulators to transcription sites. To achieve this goal, we constructed a minigene reporter system containing the genomic DNA spanning exons 26 through 29 of HDAC6. After transiently transfecting HDAC6 minigene reporter plasmid and depleting Son in HeLa cells, exons 27 and 28 were excluded in both endogenous and exogenous HDAC6 transcripts indicating the presence all the elements required to study Son-regulated splicing in the

minigene. We further tested exon exclusion in HeLa cells stably expressing our reporter construct and found that exons 27 and 28 are excluded in endogenous and exogenous HDAC6 transcripts. One important conclusion that can be drawn from these observations is that since multiple stable cell lines expressing the HDAC6 reporter minigene show the same result, there is probably no connection between chromatin context and splicing regulation by Son for splicing of exons 27 and 28 of HDAC6 transcripts.

To achieve the goal of visualizing Son-mediated splicing *in situ*, we stably transfected the HDAC6 minigene reporter in HeLa cells. However, we were unable to show the transcription locus of the minigene. Careful analysis of RNA-FISH experiments performed in parental HeLa cells show bright dots in individual nuclei that possibly could be considered as the transcription locus for endogenous HDAC6. However, the dots do not co-localize with Son and SF2/ASF consistently every time we perform RNA-FISH experiment, or in majority of cells on the same coverslip. We therefore do not consider the dots we see as transcription loci of HDAC6, but rather as an unexplained RNA-FISH signal. The next interesting observation is from RNA-FISH results of certain clones and cells transiently expressing HDAC6 reporter minigene. We observed a nuclear speckle pattern after performing RNA-FISH with HDAC6 probes. Moreover, speckle pattern is observed in parental HeLa cells hybridized with HDAC6 probe (e26-TxRed probe) which suggests a functional role of endogenous HDAC6 mRNA in nuclear speckles or post-transcriptional splicing of HDAC6 mRNA in nuclear speckles. The speckle pattern was also observed in cells that were treated for RNA-FISH but not for simultaneous immunofluorescence localization of splicing factors (data not shown) which rules out the probability of speckle pattern being a cross-talk from other channels. However, since nuclear speckles contain a large number

of different proteins and RNAs that might bind non-specifically to FISH probes, the possibility non-specific binding of HDAC6 probe to nuclear speckles could not be ruled out.

I had planned to observe alternative splicing of HDAC6 minigene *in situ* and determine the role of Son in recruiting splicing enhancers/repressors to nascent HDAC6 transcripts. Since we could not reliably visualize the transcription locus of HDAC6 reporter minigene inside the cells, these experiments could not be performed. Once the transcription locus is developed and visualized, the following experimental design can be followed to understand the effect of Son depletion in the recruitment of other splicing factors. Cy5-conjugated exon junction probes, namely EJ26/27 and EJ26/29, need to be used to visualize constitutively and alternatively spliced regions of HDAC6 minigene *in situ*. Individual probes EJ27/26, EJ29/26, E26, E27 and E29 work as controls to optimize the RNA-FISH conditions, to rule out the possibility that the experimental probes EJ26/27 and EJ26/29 bind only partially to nascent transcripts. Once hybridization conditions are set, exon skipping in control versus Son-depleted cells has to be evaluated. In a second set of experiments, performing RNA-FISH combined with immunofluorescence labeling antibodies specific to Son, SF2/ASF, SC35 and hnRNP A1 will determine how Son depletion alters their localization patterns. SF2/ASF and SC35 are nuclear speckle proteins known to promote splicing while hnRNP A1 is a splicing repressor (Sharma et al., 2010 and Millevoi et al., 2010). Performing this experiment in parallel for multiple factors, and comparing one splicing factor/repressor and Son per coverslip in both control and Son depleted cells, will allow examination of relative levels of these proteins at the HDAC6 minigene locus in control versus Son depleted cells. If SF2/ASF or SC35 decrease at the locus when Son is depleted, we can conclude that Son is required for the accumulation of these factors and that this may be important for inclusion of HDAC6 exons 27 and 28. We can also conclude that Son is a scaffolding protein

that recruits splicing factors to the transcription/splicing sites and hence when Son is absent, SF2/ASF or SC35 will not be recruited to the splice sites that influence inclusion of exons 27 and 28, resulting in exon skipping. Since hnRNPA1 is a splicing repressor, increase in its levels at the locus would suggest that presence of Son at nascent transcripts provides a signal that prevents hnRNP recruitment to promote correct splicing.

The goal of this project was to begin building reporter systems to observe Son-dependent splicing events, both at the molecular level as well as *in situ*. Since I successfully constructed and characterized the HDAC6 minigene for molecular analysis, more features can be introduced that will allow more in-depth analysis. For example, site-directed mutagenesis in ESE or ESS sequences can be introduced to learn exactly which nucleotides in the transcripts are needed for proper splicing regulation. In addition, MS2 stem loops could be introduced for purifying reporter mRNAs from control and Son-depleted cells, followed by mass spectrometry analysis to determine what splicing complexes are lost/gained from reporter transcripts in Son-depleted cells. In addition, MS2-binding protein can be introduced by transfection to directly visualize reporter mRNAs by microscopy in live cells instead of using RNA-FISH approaches. Finally, constructing a cell line similar to U2OS 2-6-3 (Janicki et al., 2004) will be needed to examine the dynamics of transcription activation and alternative splicing *in situ* on the HDAC6 reporter minigene locus.

CHAPTER 6: REFERENCES

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