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NONCANONICAL POST-TRANSCRIPTIONAL ROLES FOR SR PROTEINS DURING MICRORNA BIOGENESIS

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Thesis Abstract

Noncanonical post-transcriptional roles for SR proteins during microRNA biogenesis by Marija Dargyte

All biological processes are under regulatory control for efficient and proper homeostatic function. Dysregulation of these mechanisms results in consequences that the cell must overcome. RNA binding proteins (RBPs) are a broad class of proteins that control RNAs. They regulate all aspects of an RNA's lifecycle. The fate of RNA is dependent on interactions with its partnered RBPs. My thesis will be focused around a well characterized group of RBPs belonging to the SR protein family.

Many RBPs are first characterized based on their roles in regulation of mRNAs. However, there are many different species of RNAs created in the cell that are maintained by RBPs. MicroRNAs (miRNAs) are short non-coding RNAs that function as regulators of mRNAs. Although the main enzymes that create miRNAs have been revealed, there are still many questions as to how their synthesis is is regulated during each step of biogenesis.

In my dissertation I will show that traditionally characterized regulators of mRNA gene expression, the SR protein family members SRSF1 and SRSF3, have critical roles in miRNA biogenesis. Using a combination of in vivo and in vitro approaches we discover that these proteins bind to specific locations outside of the miRNA hairpin, a key feature required for miRNA biogenesis. This interaction promotes a structural conformation that enhances initial cleavage of miRNAs by allowing for better accessibility of the hairpin by the Microprocessor. My work expands the scope of

post-transcriptional regulation by SR proteins by defining their roles during miRNA biogenesis.

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Chapter 1: Introduction

Introduction

Gene regulation is important for maintaining the flow of genetic material in a cell. Molecular biology is centered around how DNA is transcribed to RNA, which is translated to protein. Within this dogma there are slight variations and adaptations that add to the complexity of life. For example, retroviruses do not contain DNA, rather their RNA genome is reverse transcribed upon infection of a host. Another example is that not all RNAs are translated to protein, rather RNAs such as tRNA, rRNA, lncRNA, and miRNA are functional as RNAs. Despite these incongruencies, for proper cellular function, nucleic acids and proteins have to be regulated. Disruptions in necessary gene regulation are regularly observed in cancers and diseases and must be compensated for by alternative mechanisms. In the following thesis I will explore the mechanism by which RNA binding proteins regulate noncoding RNAs.

microRNA discovery

MicroRNAs were first discovered as small regulatory RNAs that function through antisense complementarity (R. C. Lee, Feinbaum, and Ambros 1993). Ambros and colleagues made a surprising discovery that RNAs, that do not code for protein, can be regulators of development. Initial observations show the presence of two distinct length RNAs, 22 nt and 61 nt, that contain the same sequence where the 22mer was also observed to bind to the 3' UTR of the lin-4 mRNA (Lee, Feinbaum, and Ambros 1993; Slack et al. 2000; Moss, Lee, and Ambros 1997). The 22 nt and 61 nt sequences were later revealed to be mature miRNA and its precursor miRNA hairpin. Further re-

search from the Ruvkun lab describes an interaction within the 3' UTR of lin-14 gene that results in the negative regulation of lin-14 protein (Wightman, Ha, and Ruvkun 1993). Forming a model for miRNA function. A small RNA can post-transcriptionally downregulate an mRNA, through base pair complementarity found within its 3' UTR. Since their discovery, miRNAs have undergone numerous nomenclature changes, the numbers of functional miRNAs have continued to grow, and their ability for fine-tuning the transcriptome is being further studied.

microRNA function

MicroRNAs target 3' UTRs of mRNAs to negatively regulate their translational potential. Approximately 50% of 3' UTRs of human mRNA are regulated by miRNAs (Agarwal et al. 2015). Mature miRNAs function through base pair complementarity between the miRNA seed and the 3' UTR. The miRNA seed site is considered nucleotides 2-7, relative to the 5' of a mature miRNA and needs to have complete complementarity for efficient binding. Although there are differences in sequence specificity and preference for different miRNAs (Agarwal et al. 2015). The 2-7 nt 6-mer is the most common, although many miRNAs can have a preference for 2-8 when position 1 of the seed is an adenosine (Lewis, Burge, and Bartel 2005; Krek et al. 2005; Brennecke et al. 2005). Other miRNAs have requirements for 3' sequence complementarity of positions 17-22 (Lewis, Burge, and Bartel 2005; Grimson et al. 2007). Thirdly, there are some species of miRNAs that have preference for complementarity in central pairing of the mature miRNA (Shin et al. 2010). Seed sites have been observed to have variability in conservation, where conserved sites generally are more efficient sites of translational repression (Agarwal et al. 2015).

A mature miRNA is functional upon complex with the miRNA-induced silencing complex (miRISC). This complex can be composed of proteins which guide the miRNA and promote different mechanisms of translational repression. For deadenylation and ultimately 3' directed decay, GW182 is bound to miRISC which recruits CCR4-NOT, a deadenylase complex (Nakanishi 2016). The binding of this complex promotes deadenylation and decay from the 3' end of the mRNA. Deadenylation can sometimes promote decapping by DCP2 complex causing exonuclease decay from the 5' end of the targeted mRNA (C.-Y. A. Chen et al. 2009). DDX6, an RNA helicase, has been shown to be recruited to the mRNA by GW182 resulting in translational repression (Mathys et al. 2014). DDX6 is known to function as a translational repressor by interacting with translation initiation factor, eIF4E, inhibiting translation initiation (Kamenska et al. 2016).

miRNAs in cancer

Although only 22 nucleotides long at maturity, miRNAs are a conserved class of global gene regulators. Approximately 60% of human protein-coding genes contain at least one miRNA seed site (Ventura et al. 2008). Due how large the pool of miRNA targets is, it is not surprising that dysregulation of miRNAs is observed in disease and cancer. Additionally, miRNA expression profiles have been shown to be even more accurate than mRNA profiles or protein profiles to characterize cancer types (Lu et al. 2005). These expression profiles are indicative that miRNAs can function as potential therapeutics for cancer and disease progression. Similar to using antisense oligos (ASOs), miRNAs can be injected *in vivo* to target genes for regulation (Rupaimoole and Slack 2017). They also have dual roles during cancer progression. A miRNA can be oncogenic (oncomirs) if its overexpression leads to reduced expression of tumor

suppressor genes. Likewise, a miRNA can function as tumor suppressors if its expression is correlated to a decrease in anti-apoptotic genes. Two examples of such anti- and pro-oncogenic miRNAs are the let-7 family and miR-17~92 cluster.

The miRNA family let-7 consists of nine orthogonal mature transcripts. Their regulation is important during early differentiation, and dysregulation of let-7 expression has been linked to cancer initiation (Büssing, Slack, and Großhans 2008). Many of let-7 targets are oncogenes, the repressive roles let-7 imparts on oncogene expression defines this miRNA family as tumor suppressors (Balzeau et al. 2017). Reduced levels of let-7 in serums extracted from cancer patients are generally associated with a poor prognosis (Shell et al. 2007; Calatayud et al. 2017; Zhang et al. 2008). Let-7a is a well studied let-7 member and its expression has been observed to be significantly decreased in at least 12 different cancers. In ovarian cancer, decreased let-7a expression is a consequence of genomic deletion rather than insufficient processing (Zhang et al. 2008). Oncogenic genes targeted by let-7a include: MYC, LIN28, NFkB, and p53 (Saleh et al. 2011; Z. Wang et al. 2011; Viswanathan, Daley, and Gregory 2008; Iliopoulos, Hirsch, and Struhl 2009). Let-7 can also be utilized as a therapeutic to inhibit cancer progression. Overexpression of let-7 in mouse tumors has been observed to contribute to decreased tumor size and a higher survival rate (Wu et al. 2015; Esquela-Kerscher et al. 2008; Tang et al. 2016).

Likewise, the miRNA cluster miR-17~92 can function as an oncomir during cancer progression. Mir-17~92 is a group of six miRNAs that is transcribed as a single unit known as a polycistron. The genomic loci of the cluster is also observed to be over-amplified in certain cancers (He et al. 2005; Hayashita et al. 2005; Olive, Li, and He 2013). Interestingly, high expression of miR-17~92 is strongly correlated with

decreased cellular apoptosis and increased expression of MYC, an oncogene (He et al. 2005). Members of the miR-17~92 cluster have been characterized as repressors of anti-apoptotic genes. PTEN, a cell cycle regulator, is a target of miRs-19a and -19b (Jia et al. 2013). Overexpression of these miRNAs results in decreased levels of PTEN mRNA resulting in increased cell division in gliomas (Jia et al. 2013). Another miR-17~92 member, miR-92a is elevated in glioblastomas. While knockdown of miR-92a is correlated with increased cell apoptosis and BAX, a gene part of the well studied pro-apoptotic Bcl-2 protein family (Niu et al. 2012; Kale, Osterlund, and Andrews 2018). The large body of work showincasing miR-17-92 as an oncogene makes it a very good candidate for potential therapeutics.

For the context of this thesis plant miRNAs will not be discussed. Although plant miRNAs are fundamental for the plant life cycle and function as post-transcriptional gene regulators. They differ from animal miRNAs greatly (for review see J. Wang, Mei, and Ren 2019). The differences in mechanism and conservation suggest a distinct evolutionary path that gave rise to miRNAs in plants when compared to animals, thus plant miRNAs will not be considered here.

Canonical microRNA biogenesis

The majority of miRNAs are processed in a well characterized canonical pathway (Figure 1.1). A primary miRNA (pri-miRNA) is transcribed by RNA polymerase II in the nucleus (Cai, Hagedorn, and Cullen 2004). Transcriptional control of miRNAs is still relatively poorly studied. Pri-miRNAs can be found within or between protein coding genes. MicroRNA promoters and transcription initiation can be dependent or independent of surrounding protein coding genes (Ozsolak et al. 2008; Monteys et al.

2010). Using deep CAGE and Pol II ChIP-seq data Marsico et al, define transcription start sites (TSSs) of intronic miRNAs. Cap analysis and gene expression (CAGE) uses fragmented 5' ends of mRNA to identify TSSs and measure gene expression (Shiraki et al. 2003). Deep CAGE incorporates CAGE data and known transcription factor binding sites which expands the scope of the transcriptional network (de Hoon and Hayashizaki 2008). While chromatin immunoprecipitation coupled with sequencing (ChIP-seq) is a genome-wide method that reveals protein interactions with DNA (Park 2009). The authors note that more intronic miRNAs are regulated by their own promoters rather than using that of the host gene (Marsico et al. 2013). Surprisingly they observe poor or negative correlation of gene expression between the host mRNA gene and the intronic miRNA. Additionally, both exonic miRNAs and intronic miRNAs, known as mirtrons, can be transcribed by independent promoters, adding to the complexity between splicing and miRNA biogenesis (Marsico et al. 2013). Although miRNA transcripts are primarily Pol II products the transcription factors used by promoters vary greatly compared to promoters of protein coding genes (Ozsolak et al. 2008). Like protein coding genes, pri-miRNAs are 7-methylguanosine (m7G) capped and polyadenylated (Cai, Hagedorn, and Cullen 2004).

Pri-miRNAs can range drastically in length, from just over 70 nts to kilobases in length, and have relatively short half lives due to processing (Cai, Hagedorn, and Cullen 2004; Y. Lee et al. 2003). Within the long pri-miRNA transcript lies the distinct miRNA hairpin. The hairpin is typically ~70 nts in length but can vary from >100 nts to 30 nts. The hairpin possesses certain unique determinants necessary for miRNA processing, which will be discussed in more detail. The basal segments 5' and 3' of the hairpin tend to be single-stranded which promotes Drosha recognition of the hairpin (Ma et al. 2013; Jinju Han et al. 2006; Zeng, Yi, and Cullen 2005). The stem

contains an embedded mature miRNA, and is predominantly single-stranded. The apical loop contains a small stretch of unpaired nucleotides.

The initial cleavage of the pri-miRNA is performed by a complex known as the Microprocessor (Jinju Han et al. 2009; Zeng, Yi, and Cullen 2005). A minimal Microprocessor complex consists of the RNase III enzyme Drosha, and a dimerized RBP, DGCR8 (Macias, Cordiner, and Cáceres 2013). Additional cofactors and proteins such as helicases can be present in the complex to aid in processing and specificity (Mori et al. 2014). This initial cleavage event determines the mature miRNA as Drosha defines one of the ends of what will become the mature transcript (J. Han et al. 2004; Lund et al. 2004). The Microprocessor binds to the hairpin structure within the pri-miRNA transcript. This binding serves as both a ruler and guide for defining sequences within the hairpin (H. Zhang et al. 2004). Drosha cleavage occurs ~11 base pairs from the bottom fork of the stem, or basal junction (Denli et al. 2004; Ma et al. 2013). While, DGCR8 as a duplex, binds to the apical junction towards the loop of the hairpin (Kwon et al. 2016; Nguyen et al. 2015). Typical for RNase III cleavage, the cut site produces a 2 nt 3' overhang. The product of Drosha processing is known as a precursor miRNA (pre-miRNA), varying in length but approximately 70 nts long.

The Microprocessor cleavage product is known as a pre-miRNA, which is essentially the original hairpin containing the mature miRNA. The 3' overhang of the pre-miRNA serves as a signal for Exportin-5 (XPO5) binding which aids transport to the cytoplasm. When bound to RanGTP, XPO5 is able to recognize the unique 2 nt overhang found on pre-miRNAs (Du et al. 2015; Bohnsack 2004). Through electrostatic interactions with the phosphate backbone XPO5 is able to nonspecifically bind to any pre-miRNA. The complex is moved across the nuclear pore complex into the

cytoplasm where, upon hydrolysis of GTP, RAN releases XPO5 and the pre-miRNA (Lund et al. 2004; Okada et al. 2009).

In the cytoplasm the pre-miRNA is further processed by another RNase III protein, Dicer. Dicer is an endonuclease and is also responsible for the final cleavage in the small interfering RNA (siRNA) biogenesis similar to miRNA biogenesis (H. Zhang et al. 2004). Dicer contains a double stranded RNA binding domain (dsRBD), a helicase domain, PAZ domain and RNase III domains (MacRae et al. 2006). Dicer works with another RBP known as TRBP (trans-activation-responsive RNA binding protein). TRBP binds Dicer and allows for coordinated binding to a pre-miRNA. The three dsRBDs allow TRBP to bind both the pre-miRNA stem and a Dicer helicase domain (Z. Liu et al. 2018; Wilson et al. 2015). The binding of TRBP and Dicer allows for increased fidelity and flexibility of binding allowing for cleavage by Dicer (H. Y. Lee and Doudna 2012; Chakravarthy et al. 2010). Dicer is able to bind to the double stranded pre-miRNA through identification of the 3' overhang with its PAZ domain (Park et al. 2011; Tian et al. 2014). Upon binding Dicer helicase activity unwinds the hairpin and cleaves the terminal loop producing a 22 nt miRNA duplex (Park et al. 2011).

After Dicer cleavage, the resulting duplex contains the mature miRNA and its anti-sense passenger. The less thermodynamically stable strand is loaded into an Argonaute (AGO) protein family member, most commonly AGO2, while the passenger strand is degraded (Tomari et al. 2004; Khvorova, Reynolds, and Jayasena 2003; Schwarz et al. 2003; Hammond et al. 2001). Directionality determines nomenclature of the mature miRNA, 5p strand from the 5' end of the hairpin while 3p strand from the 3' end of the hairpin. Either strand can be loaded into AGO containing complex

(Khvorova, Reynolds, and Jayasena 2003). The cell can contain a major population of the mature miRNA and small population of companion strand if not degraded (Chiang et al. 2010). AGO-bound miRNA recruits additional proteins to form miRISC (Hammond et al. 2001; Mourelatos et al. 2002).

Noncanonical microRNA biogenesis

Not all miRNAs are processed through the canonical biogenesis pathway. Many of the non-canonical miRNAs arise from other noncoding RNAs, rather than bonafide pri-miRNA transcripts, although the functional relevance of these miRNAs is still being evaluated (Stavast and Erkeland 2019). Knockout experiments of major biogenesis proteins reveal that miRNAs can still be produced. Drosha, XPO5, and Dicer knockout followed by small RNA sequencing was done to see how miRNAs are processed in the absence of the canonical pathway (Y.-K. Kim, Kim, and N. Kim 2016). The authors discovered some miRNAs were still processed, suggesting alternative pathways or redundant proteins in the cell.

A major non-canonical miRNA biogenesis pathway revolves around bypassing the initial cleavage of pri-miRNAs. Drosha-independent pathways bypass the initial Microprocessor cleavage of pri-miRNAs in clever ways (Ruby, Jan, and Bartel 2007). MicroRNAs that undergo processing without Drosha are known as mirtrons (Berezikov et al. 2007). These miRNAs, found within introns, are products of pre-mRNA splicing (Okamura et al. 2007; Berezikov et al. 2007). During splicing the intron containing the miRNA (mirtron) is spliced out, and is able to function as a pre-miRNA. Mirtrons resemble unprocessed miRNAs but contain distinct characteristics (Berezikov et al. 2007). For example, mirtron hairpins are significantly

longer, and they are often 3' uridylated which requires the 3' and 5' tailing ends to be trimmed (Wen et al. 2015). Another way miRNAs form without Drosha, are co-transcriptionally (Y.-K. Kim and Narry Kim 2007; Morlando et al. 2008; Pawlicki and Steitz 2008). Nascent pol II transcripts can fold into hairpin structures co-transcriptionally and are released prematurely as ncRNAs (Pawlicki and Steitz 2008; Morlando et al. 2008). These transcripts are capped, which facilities export through the cap-binding complex-exportin 1 (EXP1) (Martinez et al. 2017). Upon transport to the cytoplasm the miRNAs are processed canonically.

Cis-regulatory elements direct microRNA Biogenesis

During the biogenesis pathway miRNAs utilize unique sequence and structural elements to modulate expression (Figure 1.2). A miRNA hairpin contains a stem and an apical loop. The stem of the hairpin is a conserved, double stranded region that can possess single nucleotide bulges.The apical loop, also known as a terminal loop, is a single-stranded, flexible, poorly conserved region within pri- and pre-miRNAs.

Most of the stem of the hairpin of miRNAs are highly complementary, it is not uncommon to see mismatches in the form of one or two nucleotide bulges. More is known about how bulges affect miRNA processing in plants. Imperfect pairing within the lower stem of *Arabidopsis thaliana* miRNAs is a driver of early miRNA biogenesis (Song, Axtell, and Fedoroff 2010). It appears that bulges are less important for processing of mammalian miRNAs. There does appear to be some sequence-specificity for bulges, and bulge localization on the stem (B. Liu et al. 2016). When examining bulge sequence variance in pri-miRNA stems, there seems to only be modest effects on processing. Furthermore, the authors of the study suggest that bulges no bigger

than 2 nt did not affect Drosha cleavage (Fang and Bartel 2015). It is unclear how bulges influence Dicer recognition and cleavage of pre-miRNAs.

Early research focused on understanding optimal sequence content and length of the loop and stem for efficient processing. A recent consensus is that a stem with a length of 36 nt is optimal (Roden et al. 2017). Some research suggests a loop of 10 nt is beneficial for Drosha cleavage, although the effects are somewhat minimal (X. Zhang and Zeng 2010; Jinju Han et al. 2006). Despite length and sequence variability not imparting a significant level of regulation, the apical loop still serves a role in recruiting auxiliary proteins.

Other determinants of miRNA biogenesis are sequence motifs. A UGU/UGUG motif can be found within the apical loop, while the stem can possess a GHG motif in the lower stem. Found within the stem GHG it is a mismatched motif, where H is any nucleotide besides guanine. GHG mismatch motif is significantly enriched in vertebrate miRNAs (Fang and Bartel 2015). The authors of the study observe enhanced processing when a GHG motif is inserted in suboptimally processed *C. elegans* pri-miR-44 *in vivo*. The 5' basal junction of the hairpin may contain UG motif (Auyeung et al. 2013). While some pri-miRNAs contain a CNNC motif within the 3' basal segment (Auyeung et al. 2013). The CNNC motif is a spatially defined, evolutionarily conserved motif, present in ~60% of pri-miRNAs. It is located 16-18 nts downstream of the 3' site of Drosha cleavage (Auyeung et al. 2013). Both the basal UG and CNNC motifs provide a single-stranded buffer (Figure 1.2). Single-stranded flanking structures are important for Drosha recognition of the hairpin and proper cleavage (Ma et al. 2013). Fang and Bartel, created artificial pri-miRNAs using known sequence determinants to observe if they are efficiently processed *in vitro*. Artificial pri-miRNAs

with only a hairpin and flanking single-stranded regions, were 6-fold less productive than the artificial pri-miRNA with all sequence motifs. These data suggest that sequence motifs are important for both Drosha and Dicer recognition and cleavage potentially functioning through recognition by auxiliary proteins.

Regulation of microRNA biogenesis by RBPs

Although the major enzymes and their binding partners of miRNA biogenesis are fairly well characterized, there are hundreds of auxiliary proteins that can bind to miRNA hairpins. Using RNA-affinity chromatography followed by mass spectrometry, Treiber et al., show that hundreds of RBPs can bind to the hairpin of pre- and pri-miRNAs. This observation expands on ways miRNAs can be regulated in different contexts and might explain different phenotypes in disease (Treiber et al. 2017). Identification of these auxiliary proteins suggests increased regulation of miRNA biogenesis. Roles for some auxiliary RBPs have already been identified.

The apical loop has been characterized as a platform for many RBPs to bind to and regulate miRNA biogenesis. The Caceres lab has published on conserved apical loops and how they can recruit proteins like splicing factor hnRNPA1 (Guil and Cáceres 2007). By binding to the UGUG motif of the apical loop of pri-miR-18a, hnRNPA1 causes the stem to undergo structural rearrangement which promotes Drosha cleavage (Michlewski et al. 2008). The authors also use complementary oligos, LooptomiRs, to bind to the apical loop, which reduces *in vitro* processing of pre-miRNAs. The authors do not distinguish if this is solely due to Dicer inaccessibility or that auxiliary proteins are no longer able to regulate processing by loop binding.

Other proteins that regulate miRNA biogenesis through apical loop binding are LIN28A and RBFOX3. Upon binding the pre-let-7a apical loop, LIN28A recruits a terminal uridylyltransferase (TUTase) which uridilates the miRNA, leading to degradation (Viswanathan, Daley, Gregory 2008; Nowak et al. 2017). RBFOX3 regulation of miRNA processing is miRNA specific. Depending on the miRNA, binding of RBFOX3 to the hairpin can either enhance or block Microprocessor accessibility to the pri-miRNA stem loop (K. Kim et al. 2014). In case of pri-miR-15a, RBFOX3 binding to the apical loop promotes Microprocessor binding and cleavage of the transcript (K. Kim et al. 2014). While with pri-miR-485, RBFOX3 binds to the stem inhibiting Microprocessor cleavage of the hairpin, yielding less transcripts (K. Kim et al. 2014).

RNA binding proteins interacting with hairpin flanking regions can also regulate miRNA processing. It is unclear how full length pri-miRNA is bound in its entirety, although a preprocessed pri-miRNA intermediate known as a progenitor (pro-miRNA) has been identified. The miR-17~92 cluster consists of seven miRNAs transcribed as a single unit (Du et al. 2015). Du et al. discovered the presence of a *cis* element 5' of the cluster that acts as an autoinhibtor of processing. This repressive domain is cleaved by an endonuclease, Cleavage and Polyadenylation Factor (CPSF3) that is recruited to the site by a poorly characterized splicing protein, ISY1 (Du et al. 2015). The cleavage product is a truncated intermediate, pro-miR-17~92, and is required for Drosha processing. This licensing step might be utilized by many different miRNAs *in vivo* but might have been missed in biochemical assays, which most likely utilize artificially trimmed or truncated pri-mIRNA transcripts. Mass spectrometry reveals 35 RBPs, mostly known splicing factors, that interact with pro-miR-17~92 suggesting the possibility for different complexes that regulate pro-miRNA generation (Du et al. 2015).

The regulation by polypyrimidine tract-binding protein 1 (PTBP1) of pri-miR-124 is another example of regulation by a sequence upstream of the hairpin. During development, PTBP1 maintains nonnerual splicing in stem cells (Shibayama et al. 2009). As cells differentiate PTBP1 expression decreases, in part due to repression by miR-124 (Linares et al. 2015). Yeom et al. discover that despite the presence of pri-miR-124-1 in mouse embryonic stem cells, mature miR-124 levels are not detectable. They show that PTBP1 binds to a pyrimidine-rich region upstream of the pri-miR-124 hairpin, which inhibits Drosha cleavage of the hairpin (Yeom et al. 2018). The authors conclude that during differentiation as miR-124 levels increase, PTBP1 is down regulated allowing neuronal specific splicing proteins to promote neuronal differentiation (Yeom et al. 2018; Makeyev et al, 2007). This feedback mechanism is an example of splicing factor regulation of miRNA biogenesis, highlighting additional roles for splicing factors in regulation of noncoding RNAs.

Another class of splicing proteins with emerging regulatory roles in miRNA biogenesis are the serine arginine-rich (SR) proteins. Primarily characterized for their roles in pre-mRNA processing, they are involved in many post-transcriptional regulatory pathways (see below). SR proteins, mainly SRSF1 and SRSF3, have been recently defined as functional in processing miRNAs (Wu et al. 2010; Auyeung et al. 2013; Kim et al. 2018).

Serine Arginine Rich Protein Family

Serine arginine-rich (SR) proteins are a family of twelve C-terminal RS (arginine serine) domain containing proteins (Table 1.1). The family is conserved throughout

metazoans, and have prominent roles in pre-mRNA splicing. Due to their essential yet redundant roles in RNA processing events, SR proteins have been widely studied the last three decades (for review see Howard and Sanford 2015).

Structural domains of SR proteins

SR proteins are characterized by their modular domain structure. A C-terminal RS domain, and phospoeptiope reactivity to monoclonal antibody mAb104 (Roth, Zahler, and Stolk 1991). As RBPs they contain at least one N-terminal RNA recognition motif (RRM). The RS domain is an unstructured, disordered region which consists of dipeptide repeats of arginines and serines which vary in lengths between proteins. Upon phosphorylation the RS domain can take on a more structured conformation, imparting stability and allowing for additional binding interactions (Xiang et al. 2013). The RS domain influences SR protein localization, specificity, and function and promotes protein-protein interactions with other RS domain-containing RBPs (Zhu 2000). Half of the SR family members contain a second RRM. SRSF1, SRSF4, SRSF5, SRSF6, SRSF9 contain an RNA recognition motif homolog (RRMH), while SRSF7 contains a zinc-binding domain, these secondary domains are separated by a linker. The RRMHs bind RNAs by interaction at an α-helix as opposed to a β-sheet, as observed with RRMs (Table 1.1). RRMHs bind RNA at reduced affinity but are necessary for specificity of the RRM (van Der Houven Van Oordt et al. 2000). The linker, RRM, and RS domain of SR proteins are subject to post-translational modifications which can influence function (Tacke, Chen, and Manley 1997; Sanford et al. 2005).

Regulation and localization of SR proteins

This multifunctional group of proteins can be regulated by post-translational modifications (PTMs). PTMs dictate SR protein localization and function. SR proteins predominantly localize in the nucleus within nuclear speckles (Cáceres et al. 1997; J. R. Sanford and Bruzik 2001). Nuclear speckles are distinguished as either interchromatin granule clusters (IGCs) or perichromatin fibrils. IGCs are believed to be the site of splicing factor storage and assembly, while perichromatin fibrils are sites of transcription of cotranscriptional pre-mRNA splicing (Saitoh et al. 2004). Phosphorylation of serines within the RS domains directs the localization and function of SR proteins. Hyperphosphorylated SR proteins are found in nuclear speckles, suggesting that phosphorylation is required for their roles during splicing (Colwill et al. 1996). SR kinases Clk/Sty, SRPK1, and SRPK2 are responsible for cooperative phosphorylation of the RS domain prior to splicing (Colwill et al. 1996; Ngo et al. 2005). During splicing, SR proteins become hypophosphorylated, where they enter a cycle of rephosphorylation to further continue splicing (Xiao and Manley 1997). SR proteins can also retain their hypophosphorylated state which allows them to stay associated with spliced mRNA and transport bound mRNA to the cytoplasm (Lai and Tarn 2004). Although SR proteins are enriched in the nucleus, SRSF1, SRSF3, SRSF4, SRSF6, SRSF7, and SRSF10 have been shown to shuttle between the nucleus and cytoplasm. Ubiquitously expressed, SR-specific protein kinases (SRPKs) can phosphorylate SR proteins in the cytoplasm as well as the nucleus (Zhou and Fu 2013). Upon phosphorylation SR proteins are recruited back to the nucleus.

SR proteins also utilize negative feedback as autoregulation of protein expression (Müller-McNicoll et al. 2019). The mechanism by which SR proteins self-regulate is known as alternative splicing coupled with nonsense mediated decay (AS-NMD). NMD is a conserved surveillance pathway that results in the degradation of premature

termination codon (PTC) containing mRNA. Different SR proteins can utilize AS-NMD with different mechanisms. SRSF1 and SRSF2 can promote splicing of introns with 3' UTRs rendering canonical termination codons useless resulting in NMD (Sun et al. 2010; Sureau et al. 2001). SRSF3-7, and SRSF9-10 can promote the inclusion of PTC containing exon. The inclusion of the exon ultimately results in NMD of the transcript (Jumaa and Nielsen 2000). Lastly, SRSF5 can autoregulate by retention of intron 5, which contains a PTC, rendering its mRNA a potential NMD target (Lareau and Brenner 2015). In addition to AS-NMD, SR proteins can utilize alternative polyadenylation and translation inhibition to autoregulate (Sanford et al. 2004; Lou et al. 1998).

Pre-mRNA splicing and SR proteins

SR proteins were first discovered three decades ago, and soon after their function as splicing factors emerged (Zahler et al. 1992). In the simplest terms, splicing is a two step transesterification reaction in which introns of a pre-mRNA are removed and the remaining exons are joined together (Berget, Moore, and Sharp 1977; Chow et al. 1977; Domdey et al. 1984; Padgett et al. 1984; Ruskin et al. 1984; Lin et al. 1985; Konarska et al. 1985). Splicing yields a mature mRNA transcript which is translated in the cytoplasm by the ribosome. Alternative splicing, where the final mRNA products will have differences in exon composition, yields alternate mRNA isoforms influence the protein sequence that is ultimately translated (Maniatis and Tasic 2002; Nilsen and Graveley 2010; Kelemen et al. 2013; Matlin, Clark, and Smith 2005). Splicing is performed by a complex macromolecular machine known as the spliceosome. The spliceosome is composed of RNAs and small nuclear ribonucleoproteins (snRNPs) which assemble and disassemble during the course of splicing (Wahl, Will,

and Lührmann 2009; Abelson 2008; Fica et al. 2013). Although the spliceosome is responsible for the catalytic steps of splicing, auxiliary proteins are responsible for guiding and defining regions to be acted on by the spliceosome.

SR proteins are well-characterized as splicing factors. *Cis*-elements within the pre-mRNA known as splicing enhancers can be found within introns (ISE) and exons (ESE). ESEs are commonly used as signals that indicate the retention of the exon and removal of the adjacent intron by the spliceosome (Schaal and Maniatis 1999; Graveley and Maniatis 1998). ESEs are recognized by SR proteins which recruit major snRNPs, U1 and U2, along with their auxiliary factors, resulting in the removal of the intron and inclusion of the ESE-containing exon (Buratti et al. 2004; Maniatis and Tasic 2002; Hertel 2008). This mechanism of exon definition is what drives different mRNA isoforms by alternative splicing.

Different post-translational modifications and structures contribute greatly to how SR proteins regulate splicing. At the beginning of splicing the RS domain of SR proteins is hyperphosphorylated, imparting rigidity. This allows for recruitment and additional interactions with other RS domain-containing splicing factors such as U2AF65 and U1 snRNP, bridging the 5' and 3' splice sites (Stark et al. 1998).

SR proteins in cancer

Most members of the SR protein family have been found to be oncogenic if the cellular context demands it. SRSF1, although necessary for maintenance of angiogenesis and tumor suppression, acts as an protooncogene when overexpressed (D. G. Nowak et al. 2010). For example, SRSF1 overexpression influences splicing of pro-apoptotic

protein, BIM. Increased levels of SRSF1 results in two isoforms that lack functional apoptotic domains resulting in inhibition of apoptosis, a hallmark of cancer (Puthalakath et al. 2007). In a similar way, SRSF3 and SRSF5 overexpression results in anti-apoptotic MCL isoform in human breast cancer cells (Gautrey and Tyson-Capper 2012). SRSF2 has been shown to regulate the transcription and splicing of RON, a protooncogene. When downregulated, SRSF2 can no longer promote the inclusion of exon 11, resulting in a constitutively expressed RON (Moon et al. 2014). Additionally, overexpression of SRSF6 results in differential expression of tumor suppressor genes and oncogenes resulting in increased cancer isoforms in both lung and colon cancer cells (Cohen-Eliav et al. 2013).

Post-splicing regulatory mechanisms of SR proteins

Six SR proteins possess shuttling capabilities, suggesting functions beyond nuclear pre-mRNA splicing. SRSF3, SRSF7, and SRSF1 have been shown to promote the transport of both spliced and unspliced genes to the cytoplasm. SRSF3 and SRSF7 specifically bind to a 22nt *cis*-element found on the unspliced histone H2a gene, which promotes export to the cytoplasm (Huang and Steitz 2001), suggesting a splicing independent role for SR proteins as guides for mRNA for translation. Post splicing, hypophosphorylated SRSF1 can interact with nuclear export protein NXF1/TAP, this interaction facilitates the export of spliced mRNA (Tintaru et al. 2007). Export of mRNA to the cytoplasm by SR proteins suggests the intriguing hypothesis that SR proteins are involved in translation.

SRSF1 has been shown to associate with translating ribosomes, perhaps influencing translation of recently spliced transcripts (Sanford et al. 2008). Furthermore, it has

been shown that SRSF1 promotes translation initiation through interactions between the m7G cap and eIF-4E by recruitment of the mTOR kinase (Michlewski, Sanford, and Cáceres 2008; Sanford et al. 2004). SRSF3 and SRSF7 have also been shown as regulators of viral translation. SRSF3 is required for viral translation by stimulating the internal ribosome entry site (IRES) leading to initiation of viral translation (Bedard, Daijogo, and Semler 2007). Hypophosphorylated SRSF7 associates with unspliced viral mRNA containing a constitutive transport element (CTE) in monosomes and light polysome fractions. This observation suggests that SRSF7 is associated with the viral transcript during export and translation initiation (Swartz et al. 2007). SRSF5 and SRSF6 have also been shown to promote translation of HIV-1 unspliced gag transcript resulting in enhanced expression of Gag protein (Swanson, Sherer, and Malim 2010). Unlike SRSF3 and SRSF7, despite translation being cytoplasmic, SRSF5 and SRSF6 regulate translation in a shuttling independent mechanism (Swanson, Sherer, and Malim 2010).

As mentioned above, SR proteins can regulate mRNA fate through AS-NMD by promoting the inclusion of PTC-containing exons. Alternatively, SRSF1 has been found to stimulate NMD by promoting UPF1 binding to PTC-containing mRNA (Aznarez et al. 2018). UPF1 is an ATP-dependent helicase required for identification and degradation of NMD transcripts (Kervestin and Jacobson 2012; Fiorini et al. 2015). The authors hypothesize that SRSF1 interacting with factors from the post-splicing complex known as the exon junction complex (EJC), is required for UPF1-dependent NMD. Typically the presence of an EJC after a PTC-containing exon results in initiation of NMD (Hug, Longman, and Cáceres 2016; Le Hir et al. 2000). SRSF1 interactions with the EJC can further enhance UPF1 binding to the NMD target resulting in increased degradation of the transcript (Aznarez et al. 2018).

SR proteins and noncoding RNAs

Although the majority of SR protein research focuses on how they regulate protein coding genes, recent data has emerged on roles in noncoding RNA (ncRNA) lifespan as well. Advances in techniques such as crosslinking and immunoprecipitation coupled to high-throughput sequencing (CLIP-seq or HITS-CLIP) have revealed novel protein and RNA interactions *in vivo* (Licatalosi et al. 2008). One such observation is that SR proteins can bind several ncRNA species including snoRNA, lncRNA, and miRNA, expanding the scope of SR protein regulation of the transcriptome.

SRSF3 and SRSF4 were shown to bind snoRNAs, suggesting that SR proteins are also involved in the removal of snoRNA introns not just mRNA introns (Änkö et al. 2012). An alternative hypothesis suggests that SR proteins interact with snoRNAs during splicing (Änkö et al. 2012). Long ncRNA, MALAT1 has been shown to regulate SR protein phosphorylation. MALAT1 guides and colocalizes with SR proteins within nuclear speckles (Tripathi et al. 2010). Likewise, A-repeats found within another long ncRNA, Xist, which is required for X-inactivation, have been shown to be required for binding and splicing by SRSF1 (Royce-Tolland et al. 2010). MicroRNAs have implicated SR proteins as auxiliary regulators during primary miRNA processing. SRSF3 has been shown to bind to a CNNC motif present on some pri-miRNAs which stimulates recruitment of the Microprocessor complex which performs the initial cleavage step in miRNA biogenesis (K. Kim et al. 2018). SRSF1 has also been implicated in miRNA processing where it binds to the miRNA-7 (miR-7) hairpin which facilitates Drosha cleavage (Wu et al. 2010).

miRNA biogenesis regulated by SR proteins

This thesis will revolve around understanding the mechanism of how SR proteins SRSF1, and to some extent SRSF3, recognize and regulate pri-miRNAs. Data derived from our research provides more context for previously published studies. As mentioned previously SRSF1 and SRSF3 have been identified as being involved during miRNA biogenesis.

A role for SRSF1, as a regulator of miRNAs was first noted a decade ago by Wu et al. Since then, little effort has been put forth to understand the mechanism by which SRSF1 acts during miRNA biogenesis. The authors of the original study observe that upon induction of SRSF1 expression, results in differential expression of 40 miRNAs is seen in HeLa cells (Wu et al. 2010). miR-7 serves as a candidate miRNA whose levels increased with SRSF1 and interestingly SRSF1 is predicted to contain a miR-7 binding site within its 3' UTR (Wu et al. 2010). The authors observe that with miR-7 overexpression, there is a decrease in SRSF1 protein levels while SRSF1 mRNA levels remain unchanged. When examining SRSF1 domain requirements for miR-7 production, there is an observed increase in miR-7 when SRSF1 contains a nuclear retention signal (Wu et al. 2010). Furthermore there is a significant decrease in miR-7 levels when the RS domain is deleted from SRSF1, suggesting the RS domain is important for miRNA processing (Wu et al. 2010). RS domains are post-translationally modified, contributing to SRSF1 shuttling, nuclear granule localization and binding interactions. Through a PCR based CLIP assay, the authors conclude that SRSF1 binds the pri-miR-7 stem region, and ultimately influences miR-7 biogenesis during Drosha cleavage. To date there has not been a protein- or RNA-dependent interaction between SRSF1 and Drosha, it is more likely that SRSF1 binds pri-miRNAs prior to

cleavage.

Meseguer et al. further explore the feedback aspect of SRSF1 and miR-10b in a neural differentiation context. The authors explore how retinoic acid (RA)-dependent neural differentiation affects miRNA expression (Meseguer et al. 2011). Upon RA treatment of neuroblastoma cells the authors saw an increase in miR-10a and -10b. They determine that SRSF1 and SRSF10 are targets of miR-10a and -10b. A negative feedback loop is drawn from this interaction (Meseguer et al. 2011). As miR levels increased, endogenous SRSF1 decreased, and vice versa. The authors also note that changes in miR-10a and -10b levels affect SRSF1 dependent splicing, favoring exclusion of previously included exons.

Recently, Xu et al has focused on SRSF1 recruitment of miRNAs to exosomes in pancreatic cancer cells (PANC1). Exosomes are secretected extracellular vesicles that contain genetic material and proteins (for review see Kalluri and LeBleu 2020). They are also utilized as communication pathways between cancer cells resulting in progression of cancer (Webber et al. 2010; Costa-Silva et al. 2015; Hoshino et al. 2015). Using a biotin labeled miR-1246, the authors capture SRSF1 as an interacting RBP (Xu et al. 2020). Through differential SRSF1 expression experiments the authors observe reduction of miR-1246 when SRSF1 expression is reduced (Xu et al. 2020), while SRSF1 overexpression results in enrichment of miR-1246 within exosomes. This mechanism of cancer progression by SRSF1 recruitment of miRNAs to exosomes underscores previous studies exploring dysregulation of SR proteins and miRNA resulting in cancer.

Our understanding of SRSF3 regulation of miRNA biogenesis has made great prog-

ress since the discovery of SRSF3 binding to the CNNC motif (Auyeung et al. 2013). Auyeung et al. demonstrate that pri-miR-16-1, which contains a CNNC motif, is processed more efficiently in the presence of SRSF3. Fernandez et al. hypothesize a mechanism by which SRSF3 regulates CNNC containing pri-miR-30c. The authors observe a genetic variant of the pri-miRNA in breast and gastric cancer; coincidentally both cancers have elevated levels of miR-30c (Fernandez et al. 2017). The variant pri-miR-30c contains a G to A mutation within the apical loop which results in a larger ssRNA bulge on the lower stem that contains the CNNC motif (Fernandez et al. 2017). This allows for increased SRSF3 binding, and increased processing. The wildtype transcript, still capable of binding SRSF3, is processed less efficiently (Fernandez et al. 2017). More recently the molecular mechanism for SRSF3 in regulating the first cleavage of pri-miRNAs was expanded (K. Kim et al. 2018). Kim et al., show that upon binding to the CNNC motif, SRSF3 can recruit Drosha to the basal junction. This interaction orients Drosha and DGCR8 in the stem of pri-miRNAs leading to the first cleavage step. The authors created a mutant pri-miR where the CNNC motif is further downstream, shifting SRSF3 binding relative to the stem. This change in position results in Drosha cleavage closer to the basal junction producing non productive miRNAs. Although the authors fail to show a direct interaction between Drosha and SRSF3 further research might even reveal stepwise interactions between SRSF3 and Drosha binding that modulate correct pri-miRNA cleavage.

It is apparent that SR proteins have a role within miRNA biogenesis, although there might be different models depending on if miRNAs are enhanced or repressed. More research is needed to tease apart the sequence determinants or motifs that guide these interactions. It is of interest to tease apart the potential feedback mechanisms axis of SR proteins regulating miRNAs, which in turn regulate SR protein coding genes.

Conclusions

As the body of research surrounding RBPs and their roles as regulators of miRNA biogenesis grows it is apparent that their mechanisms of action are complex. Although RBPs have redundant roles in binding and regulating RNA, it is clear that mechanisms behind these interactions can differ greatly.

SR proteins are an exemplar group of RBPs that have broad roles in RNA regulation. They are post-translational modified which directs how, when, and where they interact with targeted RNAs or other proteins. Additionally, SR proteins tend to interact with RNAs with broad specificity, usually in the presence of a motif with preference for ssRNA. These observations contribute to the many mechanisms of RNA metabolism that SR proteins regulate. RNA transport, subcellular localization, stability, splicing, alternative splicing, translation initiation, nonsense mediated decay, and miRNA biogenesis are regulatory pathways SR proteins have had the opportunity to regulate. Spatial and temporal interactions might differ, influencing how RNA and proteins are targeted. Thus it is important to not assume SR proteins act upon RNA in the same manner for each mechanism. In this thesis I attempt to elucidate how SR proteins deviate from mRNA regulation instead focusing on how they regulate microRNAs.

MicroRNAs are a prevalent species of ncRNAs that regulate translation of protein coding genes through specific 3' UTR interactions. MicroRNA biogenesis is a well studied pathway, with two major identified cleavage events. The emergence of regulatory interactions prior to the first cleavage by the Microprocessor is suggestive of unexplored upstream regulatory steps.

In my thesis I explore an SR protein dependent regulatory mechanism of miRNA biogenesis. I show that SRSF1 binds to a sequence 5' of the hairpin to hundreds of pri-miRNAs. I characterize that SRSF1 promotes miRNA biogenesis at the Microprocessor dependent cleavage step both *in vivo* and *in vitro*. Furthermore I determine that SRSF1 binding sites within the primary transcripts are required for SRSF1 dependent control of miRNA biogenesis. Through chemical probing experiments I show that SRSF1 is likely binding to a single-stranded region upstream of the hairpin, which is critical for proper Drosha recognition. In my third chapter I explore how SRSF1 and SRSF3 interact together with pri-miRNAs to enhance nuclear processing of the transcript. I show an SR protein concentration dependent increase of mature miRNA activity and processing. Taken together my thesis work defines a new role for SR proteins in regulating the flanking regions of pri-miRNAs.

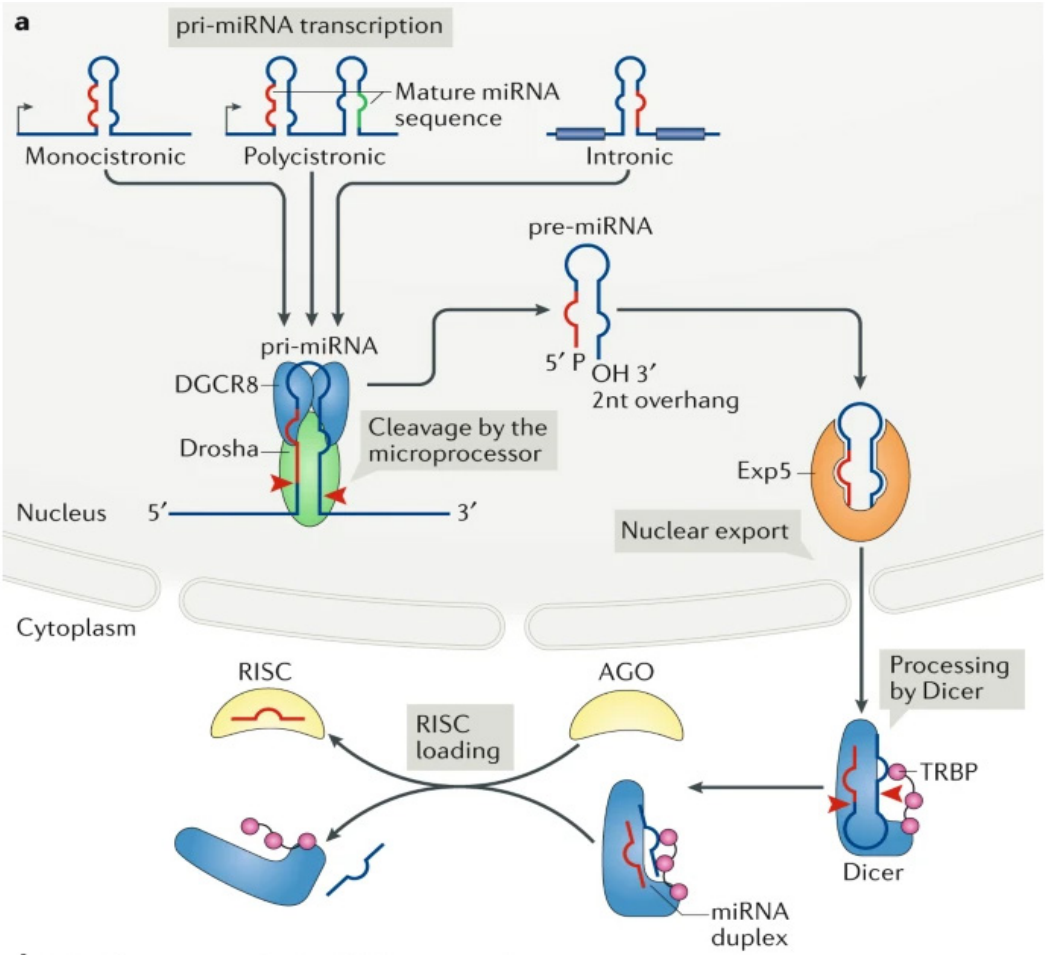

Figure 1.1. MicroRNA biogenesis pathway

Overview of canonical microRNA biogenesis. MicroRNAs can be transcribed as mono or polycistronic, as well as intronic. Primary microRNAs are initially cleaved by the Microprocessor yielding a precursor miRNA which is transported to the cytoplasm by Xportin 5. Further cleavage by Dicer yields a short micoRNA duplex, one of the strands is loaded into the RNA induced silencing complex for translational repression. Adapted from Treiber, Treiber, and Meister, 2018, Nature Reviews Molecular Cell Biology.

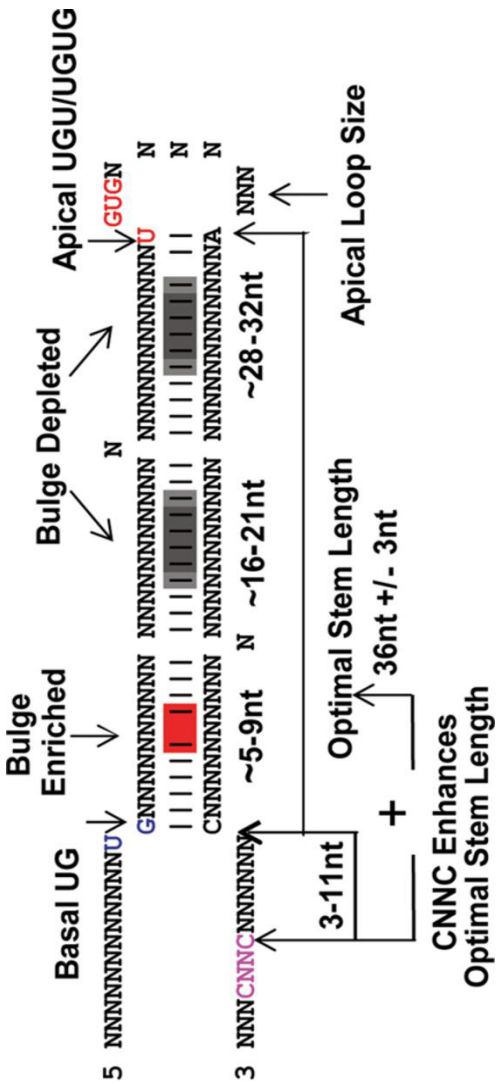

Figure 1.2. Sequence and structural determinants of pri- and pre-miRNAs

Primary microRNA containing annotations for optimal sequence and structural determinants for efficient processing. Colored sequences denote known sequence determinants: UG, UGU/G, and CNNC motifs. Within the stem red region is predicted to contain a bulge while grey regions a bulge is not preferred. Adapted from Roden et al., 2017, Genome Research.

Gene Symbol	Domain Structure	Protein Aliases	Shuttling	Molecular Functions	Biological Processes
SRSF1		**SF2, ASF, SRp30A**	**Yes**	pre-mRNA splicing; mRNA export; translation; miRNA biogenesis; mRNA stability; NMD; transcriptional elongation	apoptosis; cell-cycle; senescence; cell growth proliferation; SUMOylation; Genomic Stability; cytoskeleton organization; embryogenesis; retinal development; cardiac development; cancer
SRSF2		**SC35, SRp30B**	**No**	pre-mRNA splicing; Genomic Stability; transcriptional elongation	cell survival; cell cycle; cancer; metastasis; senescence; apoptosis; development; neural plasticity; metabolism
SRSF3		**SRp20**	**Yes**	pre-mRNA splicing; mRNA export; (viral) mRNA translation; transcriptional elongation	cell adhesion and migration; cell cycle; cell proliferation; cellular senescence; aerobic glycolysis; neuronal survival and growth; apoptosis; glucose and lipid metabolism; cholesterol homeostasis; LTM formation; development; neurological disorders; cancer
SRSF4		**SRp75**	**Yes**	pre-mRNA splicing;	neural differentiation
SRSF5		**SRp40**	**No**	pre-mRNA splicing; (viral) mRNA translation	insulin signaling; lipid transport; cell cycle; apoptosis; cancer; bipolar disorder
SRSF6		**SRp55**	**Yes**	pre-mRNA splicing; (viral) mRNA translation	drosophilia development; cardiac development; eye development; apoptosis; wound healing; cell cycle; cytoskeleton organization; genomic integrity; angiogenesis; lipid transport; muscle development; calcium metabolism
SRSF7		**9G8**	**Yes**	pre-mRNA splicing; mRNA export; (viral) mRNA processing	microtubules stabilization; viral infection
SRSF8		**SRp46**	**ND**	pre-mRNA splicing	N/A
SRSF9		**SRp30c**	**ND**	pre-mRNA splicing mRNA translation	glucocorticoid signaling; apoptosis; cell-adhesion
SRSF10		**SRp38, SRrp40**	**Yes**	pre-mRNA splicing; mRNA translation	stress response; neuronal differentiation; cholesterol biosynthesis; cell cycle
SRSF11		**p54, NET2**	**ND**	pre-mRNA splicing; genomic stability	genomic integrity; ATP synthesis
SRSF12		**SRrp35**	**ND**	pre-mRNA splicing	cell cycle

Table 1.1. SR protein family membranes and their known functions

The twelve known SR protein family members with previously used aliases. Linear RNA binding domain structure depicted for each protein. Mechanistic and biological functions are defined. Adapted from Howard and Sanford, 2016, Wiley Interdiscip Rev RNA.

Chapter 2: Splicing factor SRSF1 expands the regulatory logic of microRNA expression

Splicing factor SRSF1 expands the regulatory logic of microRNA expression

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Abstract

The serine and arginine-rich splicing factor SRSF1 is an evolutionarily conserved, essential pre-mRNA splicing factor. Through a global protein-RNA interaction survey we discovered SRSF1 binding sites 25-50nt upstream from hundreds of pre-miRNAs. Using primary miRNA-10b as a model we demonstrate that SRSF1 directly regulates microRNA biogenesis both *in vitro* and *in vivo*. Selective 2' hydroxyl acylation analyzed by primer extension (SHAPE) defined a structured RNA element located upstream of the precursor miRNA-10b stem loop. Our data support a model where SRSF1 promotes initial steps of microRNA biogenesis by relieving the repressive effects of *cis*-regulatory elements within the leader sequence.

Introduction

MicroRNAs (miRNAs) are important regulators of post-transcriptional gene expression. Nearly 60% of human protein coding genes contain conserved miRNA target sites (Friedman et al. 2009). Given the importance of miRNAs in gene regulation, it is not surprising that spatial and temporal expression patterns of miRNAs are tightly regulated. Canonical miRNA biogenesis begins with transcription of a primary miRNA (pri-miRNA) by RNA polymerase II (Lee et al. 2004). In the nucleus, the pri-miRNA folds into a hairpin structure which is excised by the Microprocessor complex consisting of Drosha and DGCR8, yielding a precursor miRNA (pre-miRNA) (Rodriguez et al. 2004; Lagos-Quintana 2003). Upon transport to the cytoplasm the hairpin is cleaved, by Dicer, into a 22nt miRNA duplex (Denli et al. 2004). The less thermodynamically stable strand is preferentially loaded into RISC by catalytic Argonaute protein, Ago2 (Noland and Doudna 2013; Park et al. 2011). Although the major catalytic steps of miRNA biogenesis and downstream RISC targeting are well understood, the regulatory checkpoints are only emerging.

RNA binding proteins are broadly implicated in miRNA biogenesis. The terminal loop region of the hairpin is a central target for many RBPs (Nussbacher and Yeo 2018; Treiber et al. 2017). For example, Lin28 binds to the terminal loop of let-7 family members recruiting TUT4 for uridylation (Heo et al. 2009). Competition between KSRP and hnRNP A1 binding to the terminal loop of pri-miR-18a influences processing by Drosha/DGCR8 (Guil and Cáceres 2007; Michlewski and Cáceres 2010). The functional importance of the terminal loop in regulation of miRNA biogenesis is underscored by strong phylogenetic conservation of this sequence element across vertebrates. In addition to the terminal loop, other sequence elements within the pri-miR-

NA are implicated in regulation of biogenesis (Michlewski and Caceres 2018).

The serine and arginine-rich (SR) protein family are evolutionarily conserved RNA binding proteins. Named for their Arg-/Ser-rich carboxyl terminal domain (RS domain), these proteins have diverse functions in post-transcriptional gene regulation including, pre-mRNA splicing, mRNA export, mRNA decay, nonsense mediated decay and mRNA translation (Howard and Sanford 2015). SR proteins are essential splicing factors and required for pre-mRNA splicing *in vitro* and *in vivo* (Zahler et al. 1993; Krainer et al. 1991; Li and Manley 2005). During spliceosome assembly, SR proteins, through the RS domain, promote splice site recognition via splicing factor recruitment (Zhu and Krainer 2000). Alternatively, the RS domain may function to promote RNA-RNA interactions by neutralizing electrostatic interactions between U snRNAs at the 5'ss and branch point sequence (Shen and Green 2006).

Previous work from our lab and others demonstrated that SR proteins interact with non-coding mRNA transcripts (Sanford et al. 2009; Royce-Tolland et al. 2010; Tripathi et al. 2010). By contrast to their roles in pre-mRNA splicing, the functional roles of SR proteins in small RNA expression remain poorly described. Two members of the SR protein family, SRSF1 and SRSF3, have been implicated in miRNA biogenesis. SRSF3 recognizes a sequence determinant located downstream of the basal junction in hundreds of pri-miRNAs (Kim et al. 2018; Auyeung et al. 2013). Whereas, SRSF1 promotes processing of pri-miR-7 by binding to the lower stem, although its mechanism remains unclear (Wu et al. 2010).

Here we report the discovery of a new sequence determinant of miRNA biogenesis. Using ENCODE eCLIP data, we discovered that a wide array of RBPs interact with

pri-miRNAs. Remarkably, we found the region 25-50nt upstream of miRNA hairpins was a frequent ligand for RBPs. Using SRSF1 as a model, we validated these data using iCLIP, which identified hundreds of pri-miRNAs in HEK293T cells with strong crosslinking signals 35-50nt upstream of the 5' end of the hairpin, which we named the 5' leader sequence. We demonstrate that SRSF1 expression levels correlate with decreased levels of pri-miRNAs and a concomitant increase in functional miRNA activity. Using pri-miR-10b as a model, we determine that SRSF1 binding sites are necessary for SRSF1-dependent stimulation of miRNA biogenesis. Taken together our data demonstrate that for the first time, an upstream *cis*-acting element recognized by SRSF1 regulates miRNA biogenesis.

Results and Discussion

Global analysis of primary miRNA-protein interactions

To identify RBPs that preferentially interact with sequences outside of the hairpin, we used the ENCODE consortium enhanced crosslinking immunoprecipitation and high throughput sequencing (eCLIP-seq) data (Van Nostrand et al. 2016). We compiled more than 120 protein-RNA interactions in HepG2 and K562 cells. We set a range to genomic regions 100nt upstream and 200nt downstream of the 5' end of pre-miRNAs, as annotated by Gencode. Using aggregated eCLIP peaks for all RBPs in the ENCODE database, we observed a wide array of interactions across pri-miRNAs, including a prominent region near the terminal loop region (Figure 2.1B). We also noted pronounced, but broadly distributed binding sites upstream of the 5' end of pre-miRNA. (Figure 2.1B) To determine how specific RBPs interact with pri-miRNAs we plotted the binding site density for individual RBPs, with binding sites in at

least 7 unique miRNAs. Unsupervised hierarchical clustering revealed that different RBPs preferentially associate with specific regions of pri-miRNAs (Figure 2.1A). For example, Lin28B interacts specifically with a region encompassing the terminal loop, a finding that is well-aligned with previous studies (Choudhury and Michlewski 2012). By contrast, we noted several splicing factors, including SRSF1 and U2AF1, with preferential binding sequences upstream of the pre-miRNA (Figure 2.1A).

Using published CLIP-seq and iCLIP experiments from our lab we validated the interaction of SRSF1 and the 5' end of pre-miRNAs (Howard et al. 2018; Sanford et al. 2009). As expected, most SRSF1 binding sites identified by CLIPper in protein coding genes were associated with exonic sequences (Figure 2.2C). We also observed a purine-rich motif enriched in sequences corresponding to SRSF1 binding sites (Figure 2.2D). At a single nucleotide resolution, crosslinking density was significantly higher in exon than intron sequences, consistent with previous studies (Figure 2.2E; (Sanford et al. 2009, 2008; Änkö et al. 2012). We used the 5' end of SRSF1 iCLIP reads to approximate the crosslinking position of SRSF1 on hundreds of pri-miRNAs (Figure 2.1C). In agreement with eCLIP data, we observed a non-uniform distribution of SRSF1 crosslinking density relative to the 5' end of pre-miRNAs, with a strong bias to positions ~50nt upstream of the 5' end of the pre-miRNA (Figure 2.1C). SRSF1 was previously linked to regulation of miRNA processing, although the mechanism was not described (Wu et al. 2010). A curious finding from the prior study was that SRSF1 recognized a consensus binding motif located in the basal region of the pre-miR-7 stem loop. By contrast, eCLIP and iCLIP show SRSF1 interacts with sequences upstream of pre-miRNAs.

SRSF1 stimulates miRNA activity

Our observations from both eCLIP and iCLIP data suggest that SRSF1 could be involved in miRNA regulation. To determine if SRSF1 has a global impact on miRNA expression, we sequenced small RNAs from control or SRSF1 overexpression cells. Of the 334 mature miRNAs expressed in HEK293T cells, we identified 21 upregulated and 17 are downregulated (Figure 2.3A, 2B). A significant majority of the differentially expressed miRNAs, 87% (33/38), were also detected in SRSF1 iCLIP experiments, suggesting they may be directly regulated by SRSF1. To further investigate the role of SRSF1 in miRNA expression we picked a handful of miRNAs of interest due to their robust SRSF1 crosslinking (Figure 2.1C). We measured changes in pri-miRNA levels bound by T7-SRSF1 in HEK293T cells by RNA immunoprecipitation (RIP) followed by RT-qPCR . We overexpressed T7 tagged SRSF1 which we immunopurfied along with any associated RNA, and then measured levels of bound pri-miRNAs. We observe significant or non-significant trends of increased pri-miRNA levels when T7-SRSF1 is immunopurified (Figure 2.1D). Pri-miR-17 and pri-miR-7, despite predicted to be associated with SRSF1 show reduction in levels by RT-qPCR (Figure 2.1D). Interestingly when looking at steady state pri-miRNA levels upon SRSF1 overexpress we observe significant reduction in expression for each pri-miRNA as compared to when SRSF1 is not overexpressed (Figure 2.3C). Suggesting that the reduced levels of T7-SRSF1 associated pri-miRNAs might be result of T7-SRSF1 overexpression. Taken together, these data suggest that SRSF1 either reduces the steady state levels of pri-miRNAs by either enhanced processing or RNA decay.

To discriminate between these two hypotheses, we asked if SRSF1 overexpression influenced mature miRNA activity. We generated luciferase reporters containing target sites for specific miRNAs within their 3'UTR. Individual miRNA reporter constructs

or a control reporter lacking the heterologous miRNA target site were co-transfected with T7-SRSF1 or a control plasmid into HEK293T cells. If SRSF1 stimulates either mature miRNA activity or expression we expect to see a decrease in reporter activity or an increase in repression. In all cases, we observed significant reduction in reporter activity relative to controls upon T7-SRSF1 overexpression (Figure 2.3D). These data suggest that SRSF1 promotes maturation of miRNAs rather than simply reducing pri-miRNA levels. To determine if these changes in reporter activity are specific to SRSF1 we also co-transfected HEK293T cells with the same reporter constructs as well as hnRNPA1, another RBP linked to the biogenesis of specific miRNAs. As expected, over-expression of hnRNPA1 enhanced miR-17 activity (Kooshapur et al. 2018). By contrast, hnRNPA1 had no effect on let-7-a1 or miR-10b reporter activity (Figure 2.4B).

SRSF1 shuttles continuously from the nucleus to the cytoplasm and is intimately involved in mRNA processing, stability and translation (Das and Krainer 2014). To determine if SRSF1 influences a nuclear or cytoplasmic step in miRNA biogenesis we co-transfected luciferase reporters with wild type SRSF1 or a non-shuttling mutant that is retained in the nucleus (Cazalla et al. 2002). If SRSF1 promotes pre-miRNA export from the nucleus or Dicer activity in the cytoplasm, then we predict that the non-shuttling mutant would be unable to stimulate miRNA activity. By contrast, we observed that relative to wild type, the non-shuttling mutant (SRSF1-NRS) exhibits enhanced repression of the miR-10b reporter (Figure 2.4C). These data suggest that SRSF1 promotes a nuclear step in the miRNA biogenesis pathway, as previously suggested by the processing of miR-7 (Wu et al. 2010).

SRSF1 binding sites are required for enhanced miR-10b activity in vivo

iCLIP revealed SRSF1 interactions with pri-miRNA 5' leader sequences at single nucleotide resolution. To determine if this broad but specific region of SRSF1 functionally relevant for miRNA processing, we created a series of deletion mutants from the 5' of pri-miR-10b (Figure 2.5A). If the 5' flanking sequences are required for mature miRNA activity, we expect an increase in miR-10b luciferase reporter activity. If the mutations remove repressive elements, we expect a decrease in luciferase activity. To distinguish between these possibilities we co-transfected expression constructs for wild type pri-miR-10b or 5' deletion mutants, along with the miR-10b luciferase reporter. We observed a significant decrease in luciferase activity for the more extreme 5'd2 and 5'd3 mutants, but not the more conservative 5'd1 mutant (Figure 2.5A). These data suggest that there are sequence or structural repressive elements within the SRSF1 binding sites 5' of the hairpin.

To determine if these points of interaction are functionally relevant for miRNA processing, we generated a series of pri-miR-10b expression constructs containing point mutations at SRSF1 crosslinking sites. If SRSF1 directly promotes miRNA biogenesis, then we predict that mutation of SRSF1 interaction sites could attenuate the effect of SRSF1 on miRNA activity and expression. As expected, driving pri-miR-10b expression up in HEK293T cells strongly reduced luciferase activity relative to the negative control expression construct (Figure 2.5B). Overexpression of SRSF1 further enhanced miR-10b luciferase reporter activity. By contrast, pri-miR-10b expression constructs containing crosslinking site mutant 2 attenuated the effect of SRSF1 on miR-10b luciferase reporters. Similarly we observe a loss of detectable mature miR-10b with mutant 2 overexpression compared to wild type pri-miR-10b (Figure 2.5B). Taken together this experiment reveals at least one *cis*-acting RNA element recog-

nized by *SRSF1 functions in regulation of miR-10b expression.*

To determine if crosslinking site mutations interfere with SRSF1 pri-miR-10b interactions, we performed filter binding assays using purified recombinant SRSF1 (rSRSF1) (Figure 2.6). We created minimal pri-miR-10b transcript variants, full length and minimal, to observe if the context of the binding site within the RNA imparts changes in affinity. The full length transcripts contain the entire hairpin along with 100nt up and downstream. The minimal construct is the string of nucleotides upstream of the hairpin containing the SRSF1 crosslinking sites, defined by iCLIP. To measure binding affinity of SRSF1 for wild type and mutant pri-miR-10b we performed filter binding assays. We calculate the fraction of bound RNA by measuring how much RNA is retained on a nitrocellulose filter compared to free RNA on a positively charged nylon filter. We observe that rSRSF1 binds full length pri-miR-10b with an apparent K_D of 27 nM (Figure 2.5C&D). Which is consistent with previously published data on SR protein affinity to pre-mRNA (Nagel et al. 1998). We observe a decrease in K_D for mutant 2 and mutant 4, 52 nM and 42 nM respectively (Figure 2.5C&D). The Hill coefficient for all the full length constructs is close to two suggesting two possible binding sites of rSRSF1, or intramolecular interactions (Figure 2.5D). Since the full length construct contains the pre-miRNA hairpin we wanted to examine how binding efficiency and saturation of the RNA changes if we only perform experiments with a minimal construct. The minimal construct is only the short region upstream of pre-miR-10b that is bound by SRSF1 *in vivo*. We observe a decrease in relative K_D for all minimal pri-miR-10b constructs (Figure 2.7). Additionally the Hill coefficient falls to just around 1 for all minimal constructs, which is lower than full length transcripts (Figure 2.7). This reduction in K_D and Hill coefficient suggests that the hairpin might impose some competition for SRSF1 binding to the upstream element. The hairpin

might also contain a second, weaker SRSF1 binding site through intramolecular interactions, presenting two SRSF1 dependent sequences for binding. We observe decreased affinity for both full length and minimal mutant 2, which we predict to be less- or non-responsive to SRSF1. Although affinity for the pri-miR-10b mutants is reduced *in vitro* we cannot discount any *in vivo* interactions that are not accounted for by filter binding, such as interactions with the Microprocessor or molecular crowding.

Identification of a repressive element in the 5' leader of pri-miR-10b mutants

To determine if the 5' leader of pri-miR-10b contains structured RNA elements we performed chemical probing using 1-methyl-7-nitroisatoic anhydride (1M7) SHAPE reagent. 1M7 modifies the 2' hydroxyl of unpaired residues. Modified ribose residues are revealed as termination sites by primer extension. Using reactive positions and lowest free energy modeling between base pair interactions we are able to derive secondary structure predictions (Figure 2.8 and Figure 2.9) (Deigan et al. 2009). We also performed a computational analysis to derive reproducibility of confidence for observed structures. Using HiTRACE as our algorithm for RNA secondary structure mapping, we are able to alter parameters that provide insight into heterogeneity of secondary structures (Yoon et al. 2011; Palka et al.). For example, titrating the slope parameter for structures produced disfavors secondary helix formation . A persistent helix at higher slope values is considered a high confidence structure. As proof of principle, the reference hairpin maintains 100% confidence despite the As expected, the regions 5' and 3' of the hairpin are reactive, indicating they are single-stranded (Figure 2.8B).

When examining the pri-miR-10b structure we observe a long high confidence hair-

pin with some reactive bulges, and a highly reactive apical loop, which aligns well with mirBase prediction of the miR-10b hairpin (Figure 2.8A&C). Single-strandedness outside of the hairpin is important for efficient Drosha binding and cleavage of the hairpin (Han et al. 2006; Zeng et al. 2005). We hypothesize that SRSF1 is binding to this region promoting single-strandness similar to how it prevents R-looping during transcription (Li and Manley 2005). Surprisingly, a 19nt hairpin emerges just upstream of the major pri-miRNA hairpin for both mutant 2 and 4 (Figure 2.10). We hypothesize that the presence of this upstream hairpin is what causes the SRSF1 decrease in affinity for the mutant transcripts (Figure 2.5C&D). We hypothesize that the upstream leader sequence is a binding platform for RBPs prior to Drosha cleavage of the pri-miRNA.

To determine if a structured 5' leader was a general feature of pri-miRNAs bound by SRSF1, we compared the thermodynamic stability of pri-miRNAs predicted to be bound by SRSF1 to those lacking iCLIP signal signal. Using the DINAmelt web server application, Quikfold, we were able to generate -dG values for predicted secondary structures of pri-miRNAs (Markham and Zuker 2005). We observed a slight, yet significant difference in the distribution of -dG between those primary miRNAs bound by SRSF1 and those that are not (Figure 2.11A). These data suggest that perhaps there is a structured element within the 5' leader sequence of SRSF1 bound pri-miRNAs.

SRSF1 promotes the first step of miRNA biogenesis

Taken together, our results suggest that SRSF1 promotes a nuclear step of miRNA processing, and likely prior to Drosha cleavage. We reasoned that SRSF1 may enhance Microprocessor complex activity. To test if SRSF1 directly influences Micro-

processor cleavage we performed *in vitro* pri-miR-10b processing assays with immunopurified Drosha/DGCR8 in the presence or absence of rSRSF1 (Figure 2.12). We measured the intensity of pre-miR-10b accumulation at different time points to see if the addition of rSRSF1 affected processing. In control reactions without rSRSF1 we observed a gradual increase in product formation over the course of a reaction (Figure 2.12, lanes 1-6). However, when pri-miR-10b was incubated in the presence of rSRSF1 we observed a higher accumulation of pre-miR-10b after 15 minutes (Figure 2.12, lanes 7-10). Since SRSF1 promotes processing by binding to the primary transcript, we hypothesize that SRSF1 can recruit Drosha to the transcript through RS domain interactions, as previously observed between other RS domain containing proteins (Boucher et al. 2001; Wu and Maniatis 1993).

To test if SRSF1 binds the Microprocessor complex we examined protein coprecipitates with Drosha by western blot. We were unable to observe any RNA-dependent or -independent interactions between exogenously expressed SRSF1 and the Microprocessor complex (Figure 2.11C). Overall, our data suggest that SRSF1 promotes pri-miRNA biogenesis by binding to the 5' leader sequence prior to Drosha cleavage.

In this study we showed that the SR protein SRSF1 promotes the first steps in miRNA processing. Global analysis of protein-RNA interactions by iCLIP and eCLIP revealed that SRSF1, as well as other splicing factors, engage binding sites upstream of pre-miRNAs (Figure 1). Reporter assays demonstrated that SRSF1 enhances miRNA function *in vivo* and that *cis*-acting SRSF1 binding sites within pri-miR-10b are required. Our data suggest that this 5' leader sequence is inhibitory, and needs to be relieved for efficient processing. Alleviating a repressive domain for miRNA biogenesis has been previously described and is well-supported by our data (Du et al. 2015).

This observation is strongly supported by *in vitro* processing assays, which show that the addition of rSRSF1 promotes early cleavage of pri-miR-10b. Coimmunoprecipitation experiments failed to detect an interaction between SRSF1 and Drosha, arguing against a recruitment model. Instead, we suggest that SRSF1 may influence the conformation of the pri-miRNA (Figure 2.13). Using SHAPE we noted the presence of a strong stem loop structure within the 5' leader region of primary miR-10b. Deletion analysis suggests the 5' leader region interferes with miR-10b expression. Taken together our data suggest that SRSF1 binding to pri-miR-10b alters the conformation of an inhibitory stem loop structure.

Despite decades of research, the mechanisms through which SR proteins regulate post-transcriptional gene expression remain unclear. Competing models include RS domain recruitment of splicing factors and RNA-RNA interaction chaperones (Graveley and Maniatis 1998; Shen and Green 2006). Previously, ATP-independent RNA annealing activity was copurified with SRSF1 (Krainer et al. 1990), suggesting that SRSF1 disrupted intramolecular RNA structure formation to promote intermolecular annealing at temperatures well below the Tm. One prediction is that SRSF1 relieves inhibitory secondary structures in the 5' leader sequence. We believe such a mechanism is consistent with our observations using pri-miR-10b as a model. This structural change could serve as a checkpoint in hairpin selection by the Microprocessor. A similar licensing step was described for processing of the pri-miR-17~92 cluster (Du et al. 2015).

The results presented here demonstrate that SRSF1 promotes miRNA processing without directly recruiting the Microprocessor. Given the recent discovery that SRSF3 influences miRNA processing through interactions with the basal junction (Kim et

al. 2018). We hypothesize that SRSF1 and SRSF3 may function collaboratively, by 5' and 3' interactions respectively, to define the hairpin for miRNA processing. This process likely involves remodeling an inhibitory secondary structure adjacent to the stem loop and consistent with an RNA chaperone function for SRSF1 in miRNA biogenesis.

Materials and Methods

Analysis of eCLIP and iCLIP datasets

eCLIP data was downloaded from the ENCODE consortium through their dashboard. Peak definitions from HEPG2 cells were aligned relative to the 5' end of miRNA precursors. Data were visualized following unsupervised hierarchical clustering. Only RBPs with at least 7 annotated binding sites near miRNAs were considered in this analysis. iCLIP data for SRSF1 was downloaded from (GSE #GSE83923). Reproducible crosslinking sites were defined as previously described (Howard et al. 2018). Crosslinking density was calculated for all SRSF1 crosslinking data relative to the 5'end of miRNA precursors.

Small RNA sequencing

For small RNA sequencing experiments total RNA was provided to RealSeq Biosciences (Santa Cruz, CA) and converted to small RNA-seq libraries using the RealSeq-AC kit. Sequencing statistics for each library are available in Supplemental Table 3.

Cell culture and transfections

Hek293T cells were grown in 6 well plates with DMEM supplemented with 10% FBS. At 70% confluence cells were transfected with plasmids using polyethylenimine (PEI) and 0.35M NaCl. Each transfection was performed a minimum of three times with two technical replicates per experiment.

RNA immunoprecipitation, RNA purification, and RT-qPCR

Whole cell extracts were isolated 24hr after transfection with RSB-100. After centrifugation, 50% of the supernatant was loaded onto anti T7 agarose beads and rotated at 4C for 1hr. After the immunoprecipitation, the beads were washed three times with RSB-100. During the last wash, the beads were treated with proteinase K (NEB) to release any bound RNA. RNA was purified using standard Direct-zol RNA MiniPrep Kit (Zymo). Final RNAs were resuspended in 15ul volumes. Equal volumes (3ul) of RNA were reverse transcribed using High-Capacity Reverse Transcriptase kit (Applied Biosystems). After reverse transcription, a 1:200 dilution was used for qPCR with Luna qPCR master mix (NEB). qPCR was performed using Luna Universal qPCR Mix (NEB) and performed on QuantStudio 3 Real-Time PCR System (Applied Biosystems) according to MIQE guidelines (Bustin et al. 2009).

Luciferase reporter assays

Seed sites for let-7a-1, miRs -1, -7, -9, -17, -100 were inserted into the 3'UTR of pMIR-REPORT (Life Scientific). miR-10b reporters described previously (Ma et al. 2007) were obtained from AddGene. Reporters were co-transfected with Renilla luciferase (Promega) reporter as a transfection efficiency control. Luciferase activity was

assayed 24 hours post transfection using Dual-Glo Luciferase Assay System (Promega). For a 24-well plate, each well was transfected with 100ng of TK-rLUC (Promega), 800ng or 1ug of T7-SRSF1 or control plasmid (Cáceres et al. 1997), 400ng of pMIR Luciferase reporter (Life Scientific). Experiments with exogenous pri-miRNAs, cells were transfected with 200ng of pGK (control) or experimental pGK plasmids (Ma et al. 2007; Cáceres et al. 1997).

In vitro transcription

Oligos were designed containing pri-miR-10b transcripts with a T7 site using Primerize online tool and prepared using standard fragment PCR assembly Primerize protocol. 8pmol of PCR products were *in vitro* transcribed using alpha-32P UTP and MEGAscript T7 polymerase kit (ThermoFisher). Following transcription, RNA was phenol/chloroform extracted and ethanol precipitated. RNA was resolved on a 6% denaturing polyacrylamide gel and extracted with a clean razor. RNA containing gel was incubated overnight at 42ºC in elution buffer (0.3M NaOAc pH 5.5, 2% SDS). RNA was ethanol precipitated and stored at -20ºC until use.

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Author Contributions

JRS conceived the study. JRS, MD, JP, CDP, MDS. designed the experiments. MD, CDP, JP. performed the experiments. MD, CDP, JP analyzed the data. JRS and MD. wrote the manuscript.

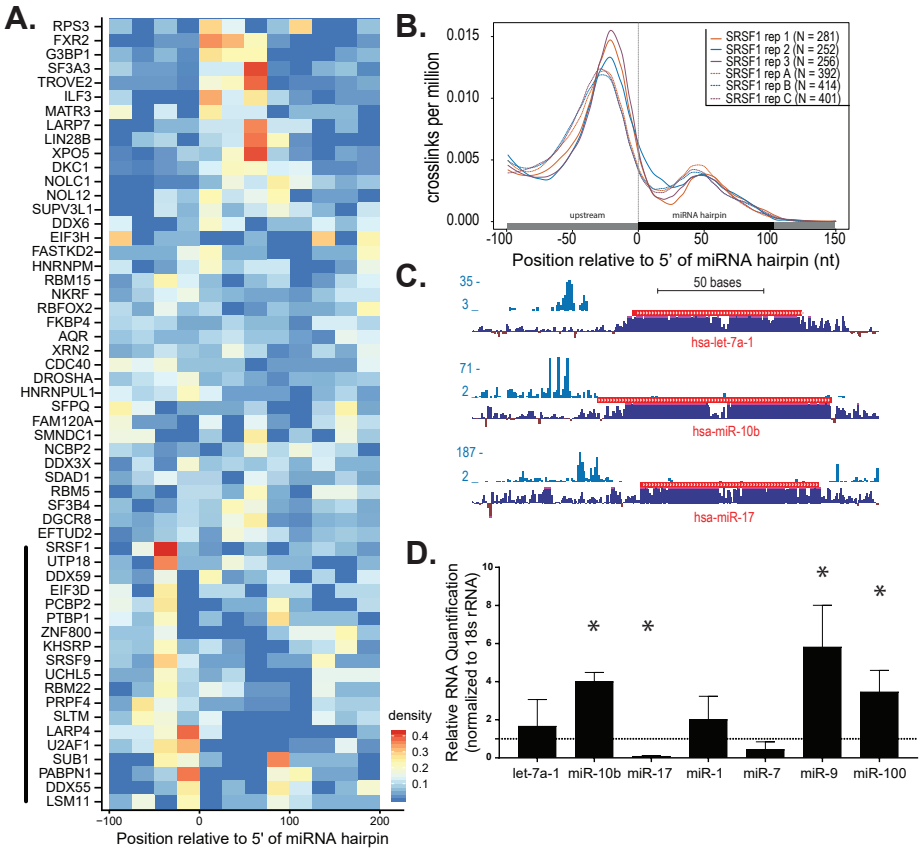


Figure 2.1. Meta analysis of eCLIP data and iCLIP data characterizes a relationship between RBP binding and pri-miRNAs. (A) Heatmap depicting specific RBP interactions along a subset of pri-miRNA transcripts using HepG2 eCLIP data. Horizontal axis denotes distance from the 5' end of miRNAs by bins in 25nt. (B) SRSF1 iCLIP crosslinks density relative to pri-miRNA for six replicates under two conditions. (C) UCSC genome browser screenshots of three exemplar pre-miRNAs with SRSF1 binding. Blue histogram is SRSF1 crosslinking density. Red track is pre-miRNA genes. Purple histogram is 100 vertebrate conservation. (D) Relative quantification of T7-RIP RT-qPCR normalized to 18s rRNA for respective miRNAs with control or SRSF1 overexpression. IP samples were normalized to respective input (dashed black line). Multiple unpaired, nonparametric t-test (*) $P < 0.05$.

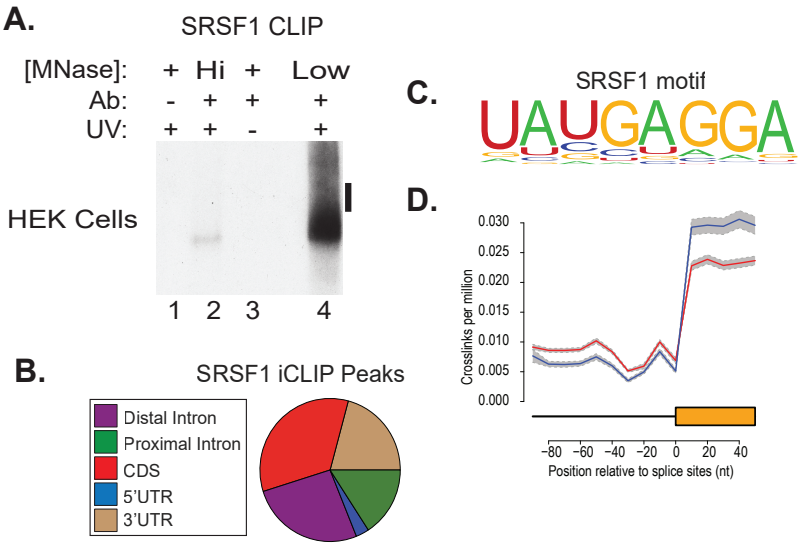


Figure 2.2. SRSF1 iCLIP results. (A) Autoradiograph of protein-RNA complexes. Black line denotes where within the smear in lane 4, protein-RNA complexes were excised. (B) Pie chart denoting where SRSF1 iCLIP peaks map back to the genome. (C) Consensus motif for SRSF1 derived from above iCLIP. (D) Graph depicting SRSF1 crosslinking sites relative to intron-exon junctions. Note SRSF1 crosslinks higher over exons. Blue line is SRSF1 crosslinks from SRSF1 overexpression background, while red line is SRSF1 crosslinks from a control cell line.

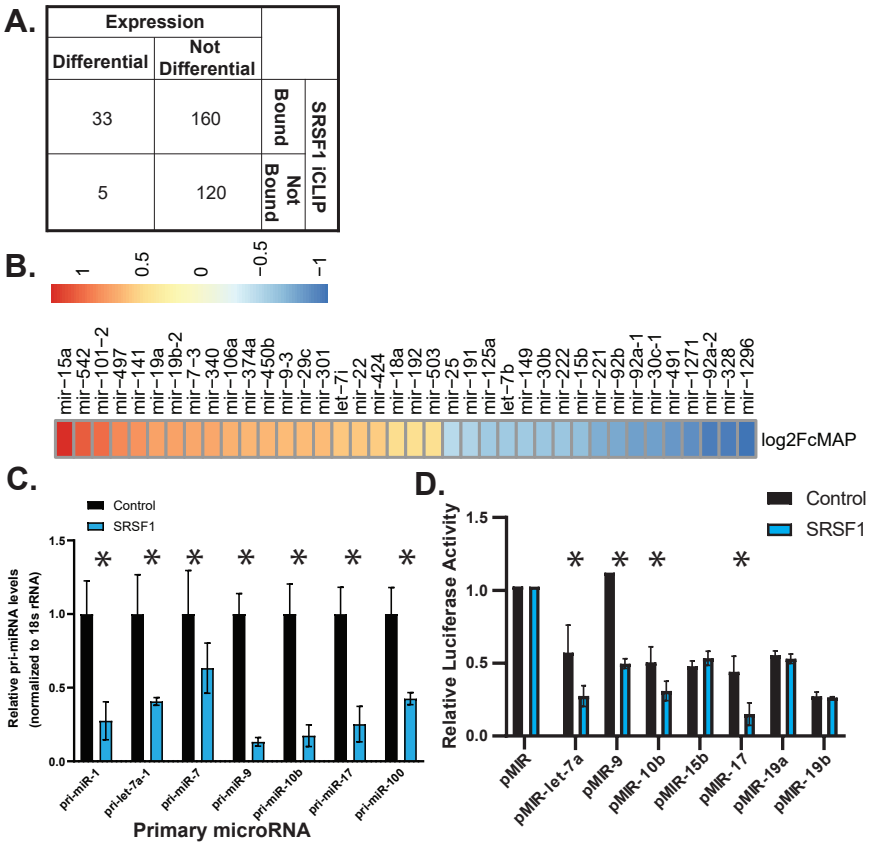


Figure 2.3. (A) Table depicting degree of overlap between miRNAs identified by smRNA-seq and iCLIP. (B) Heat map showing miRNAs as defined by MirGeneDB 2.0 that are up (red) and down (blue) regulated after SRSF1 overexpression. (C) Relative quantification of RT-qPCR of pri-miRNAs normalized to U6 rRNA in control and SRSF1 overexpressed cells. (*) P <0.05 using unpaired t-test. (D) Luciferase reporter activity of reporters containing respective mature miRNA seed sites along with SRSF1. (*) P <0.05 using unpaired t-test.

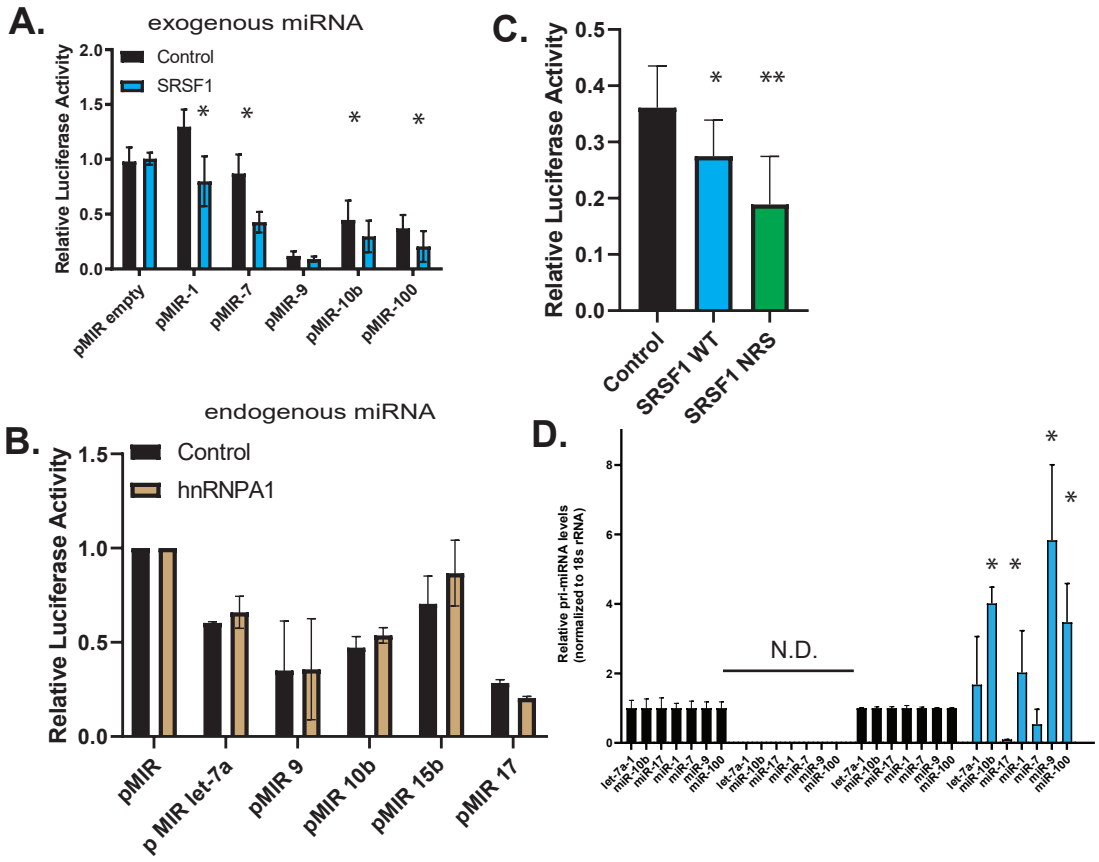
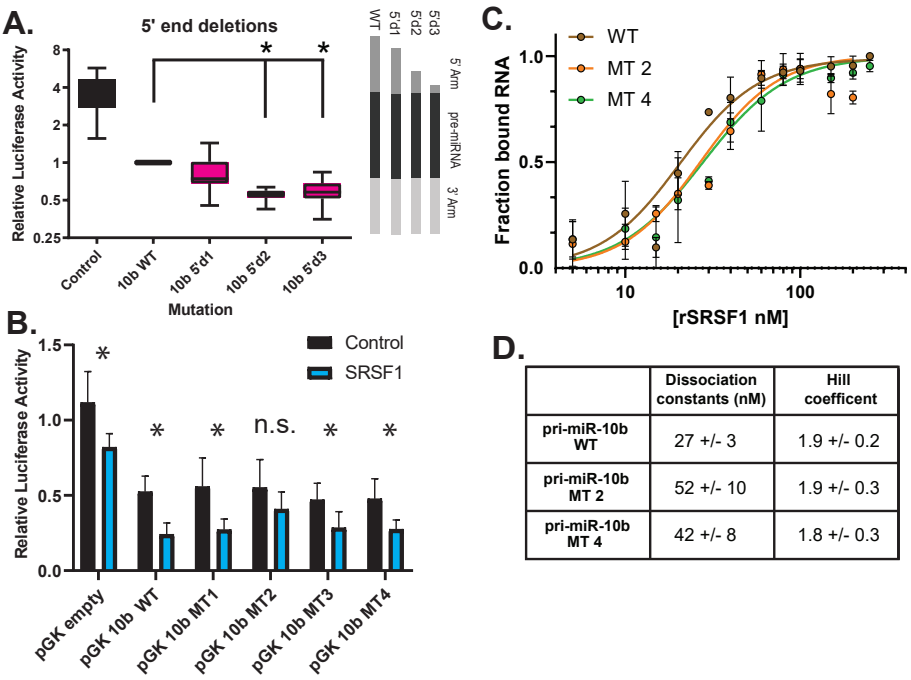


Figure 2.4. *in vivo* miRNA activity and expression under different conditions (A) Relative luciferase reporter activity for reporters containing respective mature miRNA seed sites with exogenous miRNA transfection and SRSF1 overexpression (blue). Multiple unpaired, nonparametric T-test (*) < 0.05. (B) Relative luciferase reporter activity for reporters containing respective mature miRNA seed sites with hnRNP A1 overexpression (brown). (C) Relative luciferase activity for miR-10b for control (black), SRSF1 (blue), and SRSF1 NRS mutant (green) overexpression. Unpaired, nonparametric T-test (*) < 0.05. (D) Relative quantification of T7-RIP RT-qPCR data for respective miRNAs with control or SRSF1 overexpression. IP samples (blue) were normalized to input (black), control IP did not detect enrichment of any pri-miRNA. Multiple unpaired, nonparametric T-test (*) < 0.05.



	Dissociation constants (nM)	Hill coefficent
pri-miR-10b WT	27 +/- 3	1.9 +/- 0.2
pri-miR-10b MT 2	52 +/- 10	1.9 +/- 0.3
pri-miR-10b MT 4	42 +/- 8	1.8 +/- 0.3

Figure 2.5. Mutations within SRSF1 binding site alter miR-10b expression and activity. (A) Relative luciferase activity of miRNA-10b with overexpression of exogenous pri-miRNA-10b transcripts containing 5' truncations. (*) P <0.05 using unpaired t-test. (B) Luciferase reporter activity for miR-10b reporter when exogenous pri-miR-10b mutants are coexpressed with SRSF1 overexpressed cells. (*) P <0.05 using unpaired t-test. (C) Wild type and mutant pri-miR-10b binding to SRSF1. Tables list relative KDs and Hill coefficients for wild type or mutant pri-miR-10b from filter binding assays. KDs reject null hypothesis using one-way ANOVA. Using t-Test when comparing WT to MT 2 (p-value=0.001) WT to MT 4 (p-value=0.004) MT 2 to MT 4 (p-value=0.819).

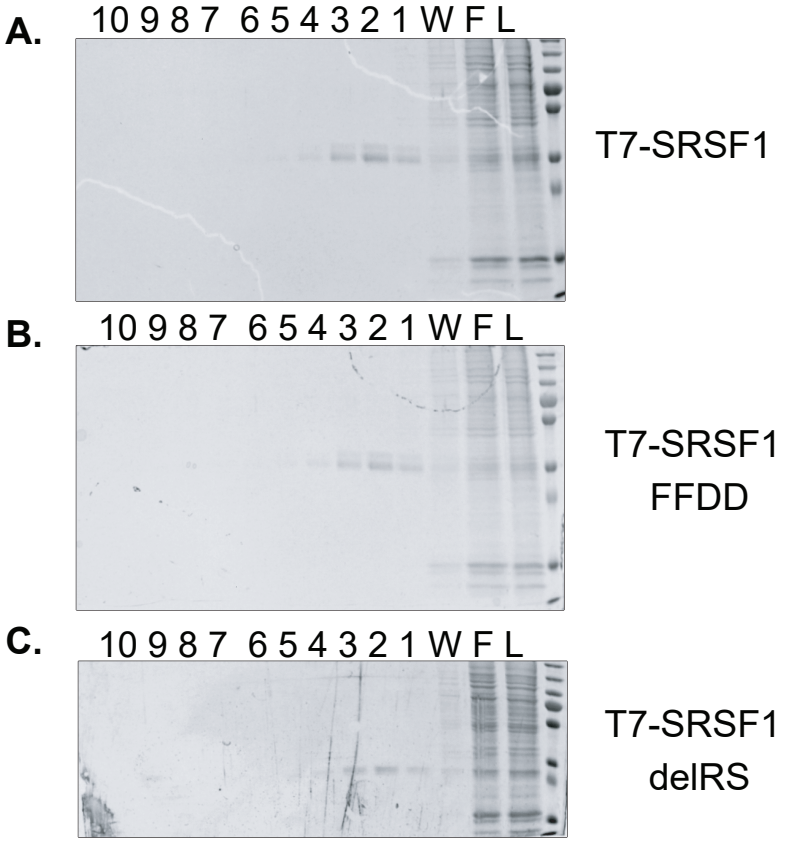
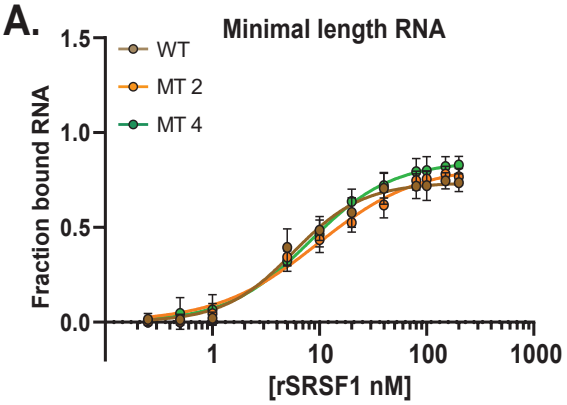


Figure 2.6. Purification of T7-SRSF1 and mutants from HEK293T cells. Commasie stained gels from SRSF1 (A), SRSF1-FFDD (B), and (C) SRSF1 delRS. From right to left L is lysate, F is flow through, W is wash, 1-10 are 10 serial elutions. Two of the highest intensity elutions were collected for dialysis.

A.



B.

	Dissociation constants (nM)	Hill coefficent
pri-miR-10b WT	6.5 +/- 3	1.3 +/- 0.2
pri-miR-10b MT 2	9.2 +/- 10	0.9 +/- 0.1
pri-miR-10b MT 4	8.5 +/- 8	1.1 +/- 0.1

Figure 2.7. Quantified filter binding assay measuring the fraction of 0.25nM RNA bound by recombinant SRSF1. (A) Minimal constructs of wild type and mutant pri-miR-10b binding to SRSF1. The minimal constructs only include the length of sequence that is bound by SRSF1 defined by iCLIP. Non-linear regression was fit assuming one binding site for SRSF1 on pri-miR-10b. (B) Table lists relative KDs and Hill coefficients for wild type or mutant pri-miR-10b from filter binding assays above.

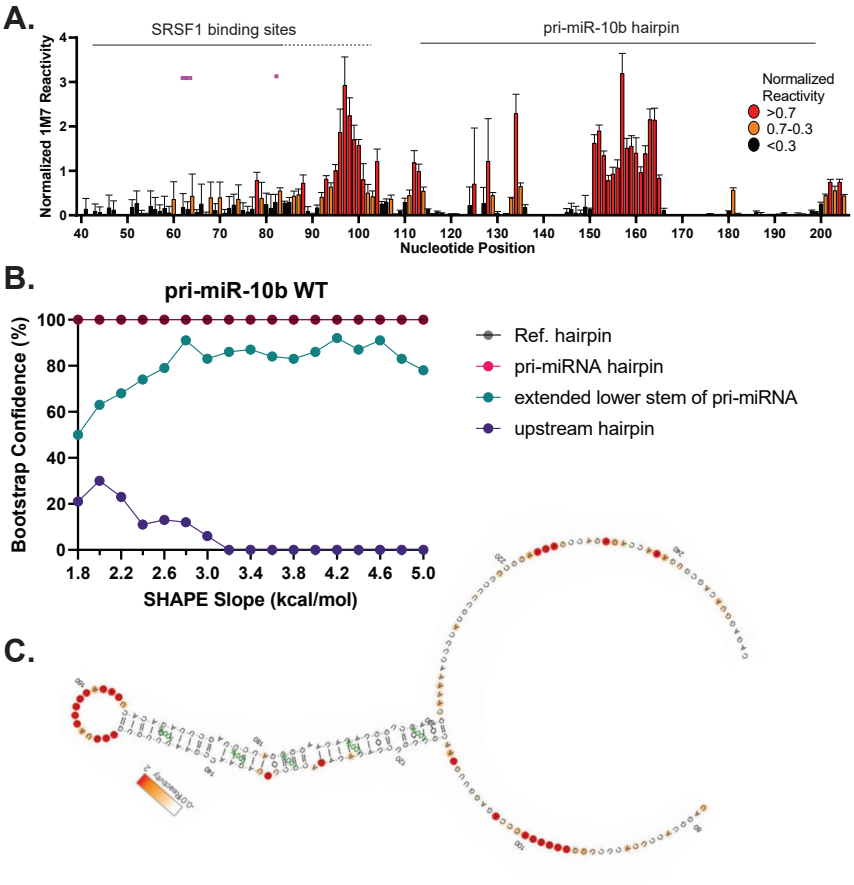


Figure 2.8. Secondary structure of pri-miR-10b by 1m7 chemical probing. Structures for wild type pri-miR-10b. (A) Normalized 1M7 reactivity for nucleotide positions 40-220 of the pri-miR-10b construct. Regions containing SRSF1 cross-linking sites, and pri-miRNA hairpin are labeled in black solid lines. Upstream hairpin visible in mutant constructs is annotated with dashed black lines. Regions of mutations are labeled with purple lines mutant 2 mutation is 62-64nts, mutant 4 single mutation is at 82nt. Nucleotides are color coded based on their normalized average reactivity. Experiments were performed three times with three technical replicates. (B) 100 bootstrap replicate confidence percentage for structures calculated by RNAstructure with increasing SHAPE slope parameter. Observed structures include: the reference hairpin, pri-miRNA hairpin (from 115-150nt), extended bottom of pri-miRNA hairpin of a 6bp stem and 4nt bulge (from 105-114nt), and an upstream hairpin (from 75-96nt). (C) Predicted secondary structure of wildtype pri-miR-10b. A threshold of 55% bootstrap confidence was used to remove low confidence structures. Warmer colors denote higher 1M7 reactivity at a particular nucleotide position.

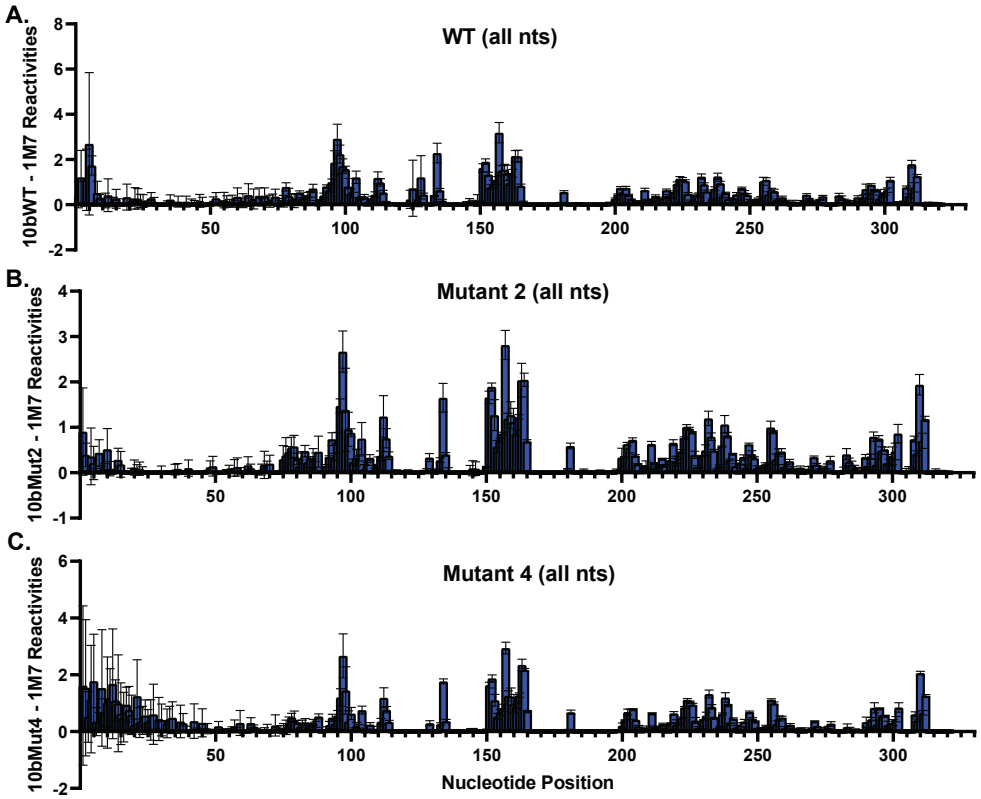


Figure 2.9. Chemical mapping of 1M7 reactivities for full length constructs and slope titrations for pri-miR-10b mutants by SHAPE. Wildtype (A), mutant 4 (B), and mutant 2 (C). Plots show normalized reactivities where each reactivity was generated as an average of three technical replicates from three separate probing experiments.

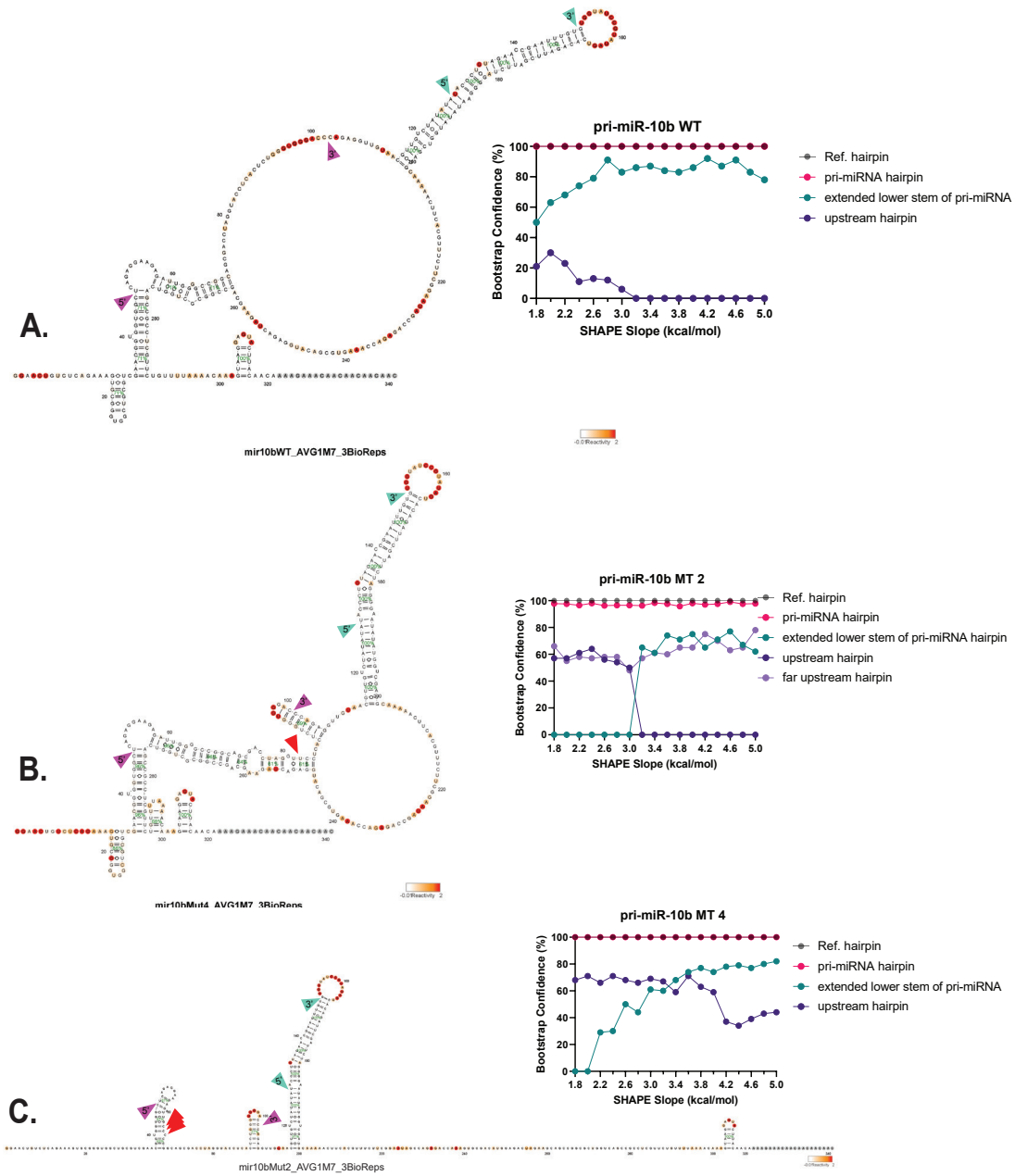


Figure 2.10. Secondary structures of pri-miR-10b by 1M7 chemical probing. Structures for wild type pri-miR-10b (A), mutant 4 (B), and mutant 2 (C). Arrows distinguish important features of the pri-miRNAs. SRSF1 cross linking parameter (purple) and embedded mature miR-10b (cyan). Red arrows denote sites of substitution mutations for the individual mutations. Note boxed, the presence of a small and stable hairpin upstream of the 5' apical stem of miR-10b. The color bar denotes nucleotide reactivity. Accompanying plots show normalized reactivities where each reactivity was generated as an average of three technical replicates from three separate probing experiments. HiTRACE slope parameter titrations

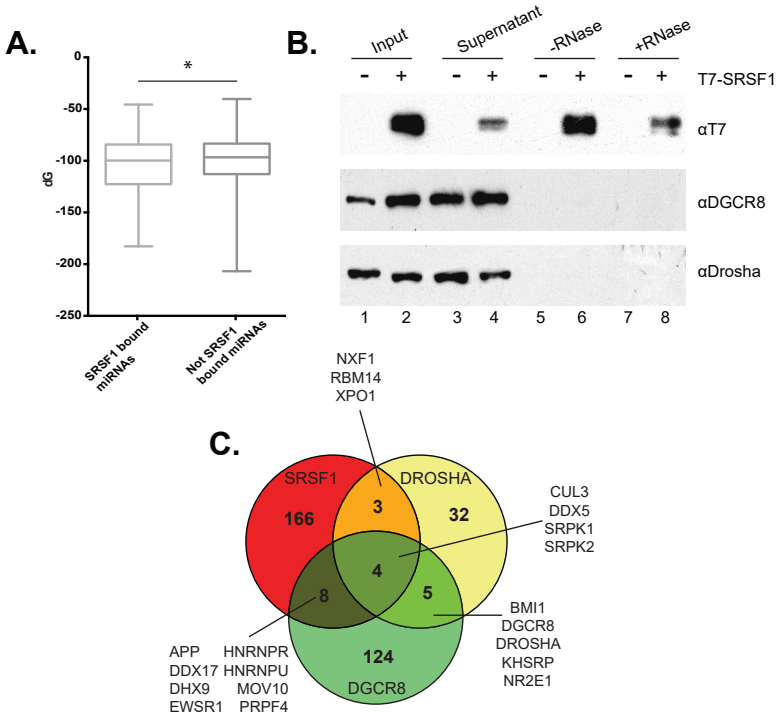


Figure 2.11. Predicted global RNA structures and protein-RNA interactions. (A) Predicted Gibbs free energies calculated for pri-miRNAs either bound or not bound by SRSF1, as defined by iCLIP. (*) P <0.05 using unpaired t-test. (B) Western blots for T7, Drosha, and DGCR8 from T7 immunoprecipitation. Control or T7-SRSF1 overexpressing cells were immunoprecipitated followed by RNase digestion (lanes 5-8). (C) Protein-protein interactions for Drosha, DGCR8, and SRSF1 attained from BioGRID.

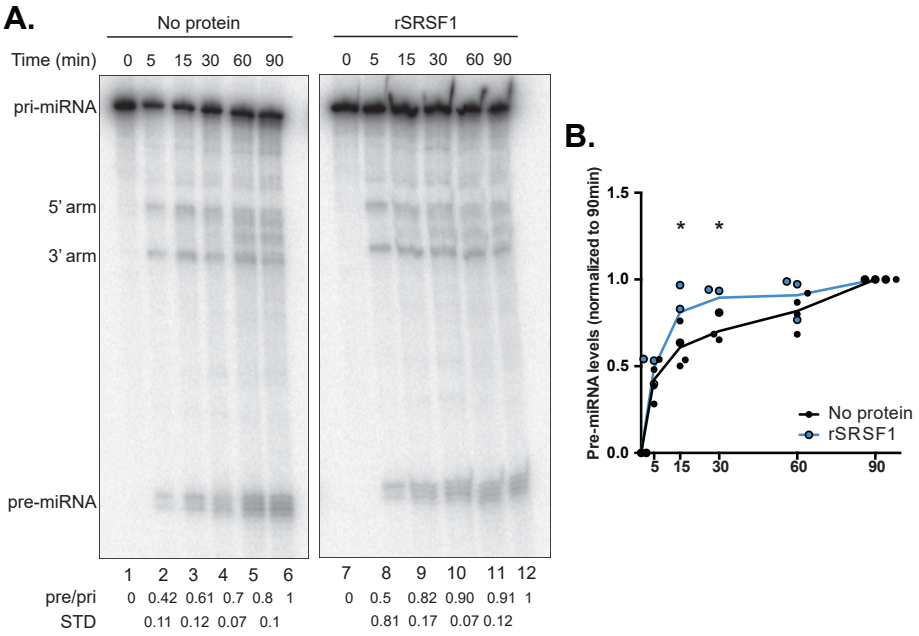


Figure 2.12. SRSF1 directly alters the rate of pri-miRNA processing. (A) In vitro processing time course of pri-miR-10b constructs by FLAG pulldown Microprocessor complex in presence or absence of rSRSF1. 5' or 3' arms cleaved during processing are labeled. Pri- to pre-miR-10b ratios are calculated for three replicate experiments. (B) Quantification of pre-miR-10b accumulation over 90 minutes. Significance at 15 and 30 minutes were calculated by unpaired T-test (*) < 0.05.

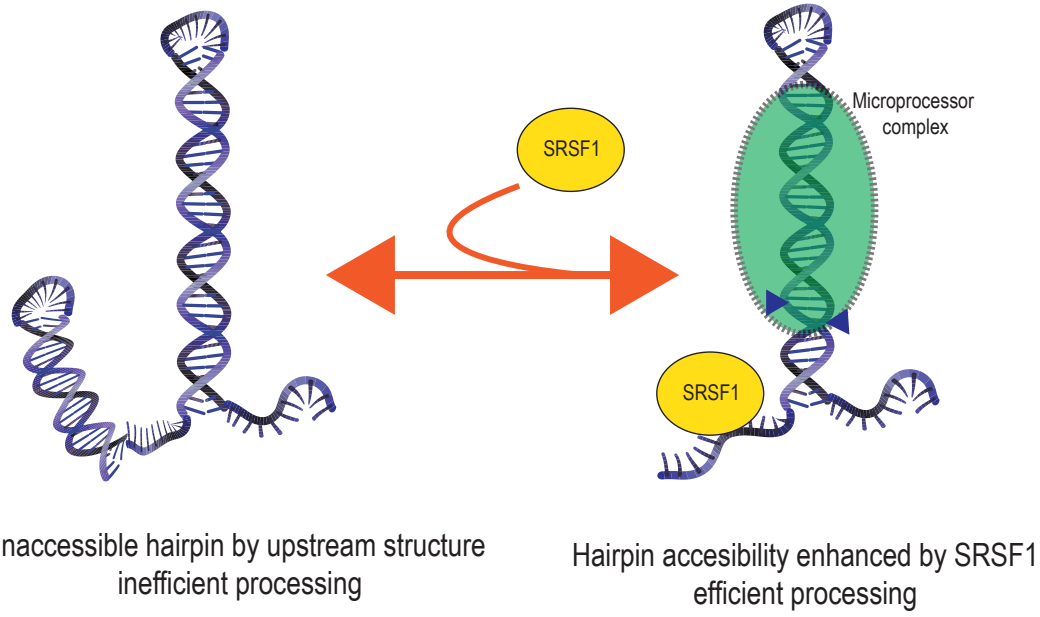


Figure 2.13. Model for SRSF1 enhancing miRNA biogenesis. Sequences upstream of the pri-miRNA hairpin may exist as either structured or unstructured. A structured element results in reduced accessibility of the hairpin by the Microprocessor complex. SRSF1 is binding upstream of the hairpin promoting single-strandedness allowing for efficient recognition and cleavage of the hairpin.

Chapter 3: Co-regulation of miRNA biogenesis by SRSF1 and SRSF3

Abstract

The serine and arginine-rich splicing factors, SRSF1 and SRSF3 are key regulators of gene expression. We recently discovered a role for SRSF1 in positively regulating miRNA biogenesis through interaction with a region upstream of the pri-miR-10b hairpin. SRSF3 regulates miRNA biogenesis through interactions downstream of hairpin at the conserved CNNC motif. We hypothesize that SRSF1 and SRSF3 interact together to cooperatively promote the processing of pri-miRNA hairpins. To test this hypothesis we analyze previously published CLIP data sets to identify miRNAs bound by both proteins. We perform filter binding assays to characterize the affinity of SRSF3 for pri-miR-10b. Structure probing of SR targeted transcripts reveals possible secondary structures flanking the pri-miRNA hairpin that might impede the initial processing event. Using *in vivo* reporter assay we observe enhanced activity of mature miR-10b when both SR protein levels are increased suggesting they cooperatively regulate processing *in vivo*.

Introduction

MicroRNAs (miRNAs) are a species of small noncoding RNAs that regulate post-transcriptional gene expression. They function by base pairing to the 3' UTR of mRNAs resulting in translational repression or mRNA decay. MiRNA target sites are highly conserved indicating their importance across evolution (Agarwal et al. 2015). In addition to their conservation, miRNAs function throughout many processes of cellular growth, including developmental pathways and cellular maintenance (Bracken,

Scott, and Goodall 2016). Dysregulation of miRNA expression is observed in many human diseases and correlated in different cancer types (Ventura et al. 2008). Due to their roles in both normal and diseased cells it is important to understand how miRNAs are regulated.

MicroRNA biogenesis begins in the nucleus where a primary transcript (pri-miRNA) is transcribed by Pol II. Pri-miRNA transcripts vary in length from several hundreds to thousands of nucleotides. Embedded within the pri-miRNA is the hairpin which contains a stem-loop structure of 60-80 nts. The hairpin is excised by the Microprocessor complex consisting of Drosha, and DGCR8. This cleavage product,or precursor miRNA (pre-miRNA), is then transported to the cytoplasm where it is further cleaved by the RNase III enzyme, Dicer. Dicer generates a short, 22 nt duplex that consists of a mature miRNA and its antisense pair. The antisense strand is degraded while the mature miRNA is bound by an Argonaute protein. Argonaute bound miRNA complex is known as the RNA induced silencing complex (RISC), which targets 3' UTRs of protein coding genes for translational repression. Although the enzymes involved in miRNA processing have been identified and characterized it is clear that many auxiliary proteins interact with both pri- and pre-miRNAs (Treiber et al. 2017).

The Serine Arginine-Rich (SR) proteins can also promote miRNA processing (Wu et al. 2010; Dargyte et al. n.d,; Auyeung et al. 2013; K. Kim et al. 2018). By contrast to their well established roles in pre-mRNA splicing, their regulation of miRNA processing is an emerging area of research. We recently discovered a global role for SRSF1 in miRNA processing by binding to an element 5' of the pri-miRNA hairpin. We demonstrate that removal of this upstream element allows for efficient miRNA processing. Similarly, SRSF1 binding to this element enhances pri-miRNA cleav-

age at the Microprocessor dependent step. SRSF3 also regulates miRNA biogenesis. Auyeung et al. discovered that SRSF3 binds to a conserved CNNC motif present in 60% of human pr-miRNAs. By contrast to SRSF1, the CNNC motif is located 13-17 nts downstream of the pri-miRNA hairpin (Auyeung et al. 2013). Upon binding to the CNNC motif, SRSF3 promotes association of Drosha to the hairpin, enhancing processing of the transcript (K. Kim et al. 2018). One gap in the field is the lack of interaction between the Microprocessor or any SR protein, which could indicate that SR proteins bind a step prior initial cleavage of pri-miRNAs. Alternatively SR proteins could influence processing by promoting a favorable RNA conformation.

During regulation of spliceosome assembly, SR proteins function in context dependent and combinatorial mechanisms. SR protein interactions with each other can influence their roles during splicing. During pre-mRNA splicing SR proteins can coordinate binding to exons which regulates alternative exon inclusion or exclusion (Pandit et al. 2013). Positional regulation of SR proteins on pre-mRNA transcripts reveals that SR protein binding is dependent on the presence or absence of other SR proteins (Howard et al. 2018; Pandit et al. 2013). We hypothesize that SR proteins co-regulate miRNA biogenesis by coordinated binding on the flanking regions of a pri-miRNA hairpin.

In this study, we test the hypothesis that SR proteins co-regulate miRNA biogenesis. Using miRNA-10b (miR-10b) as our model we demonstrate that SRSF3 can bind pri-miRNAs at regions flanking the hairpin, but also within the hairpin. *In vitro* and *in vivo* experiments reveal that the presence of both SR proteins promotes miRNA biogenesis.

Results

SRSF3 binds pri-miRNAs on a global scale

We previously demonstrated that SRSF1 binds upstream of pri-miRNA hairpins (Dargyte et al. n.d.). To determine where SRSF3 binds pri-miRNAs we analyzed previously available protein-RNA interactions data (Anko et al. 2012; Xiao et al. 2016). SRSF3 crosslinking and immunoprecipitation (CLIP) was performed in either mouse embryonic carcinoma, P19 cells, or huaman cervical cancer HeLa cells. We observe that a majority of SRSF3 P19 CLIP counts relative to the pri-miRNA sequence, are outside of the hairpin region (Figure 3.1A). SRSF3 binding 3' of the hairpin is expected (Auyeung et al. 2013). Surprisingly, the region 100 nts upstream of the hairpin contains more SRSF3 counts than 3' of the hairpin. CLIP counts from. CLIP data from HeLa cells reveals that SRSF3 binds within the pri-miRNA hairpin (Figure 3.1B). Interestingly, we observe that 66% of SRSF3 bound miRNAs bind the mature miRNA sequence within the hairpin (Table 3.1). Suggesting that SRSF3 might have a role in regulating pri-miRNAs independent of the downstream CNNC motif by binding upstream within the hairpin.

SRSF3 decreases pri-miRNAs expression in vivo

To validate SRSF3 CLIP data we performed an RNA immunoprecipitation (RIP), using exogenously expressed T7 epitope tagged SRSF3. Following overexpression of T7-SRSF3 in HEK293T cells we performed an immunoprecipitation using an antibody against the T7 epitope (Figure 3.3A). Protein bound RNA was released by proteinase K digestion, and RNA was characterized by RT-qPCR (Figure 3.3B). Com-

plimentary SRSF1 data from Dargyte et al. is presented as a comparison. Despite let-7a-1, miR-7, miR-10b, and miR-100 also being shown to interact with SRSF3, we observe that pri-miR-1 is the only miRNA that is significantly enriched with T7-SRSF3 (Table 3.1 and Figure 3.2B). The RIP was performed in an SRSF3 overexpression background. We wanted to determine if T7-SRSF3 overexpression influences endogenous pri-miRNA levels, which might explain reduced precipitation of T7-SRSF3 with expected pri-miRNAs.

To determine if SRSF3 affects miRNA expression we measured pri-miRNA levels after T7-SRSF3 overexpression. Whole cell RNA was extracted and pri-miRNA levels were assessed by RT-qPCR. Similar to steady state pri-miRNA levels when SRSF1 is over expressed, we observe a significant decrease in pri-miRNA levels when SRSF3 is overexpressed (Figure 3.2C). A decrease in pri-miRNA levels can explain the lack of association between pri-miRNA and SRSF3 observed with the RIP. The decrease in pri-miRNA levels could be a result of processing or degradation of transcripts. We wanted to further characterize the interaction between pri-miRNAs and SRSF3.

SRSF3 interacts with pri-miR-10b in vitro

To quantify SR protein interactions with pri-miRNAs *in vitro* we performed filter binding assays. For our study we purified recombinant SRSF1 and SRSF3 (rSRSF1 and rSRSF3) from HEK293T cells (Cazalla et al. 2005). Since SRSF1 and SRSF3 both interact with pri-miR-10b we wanted to biochemically characterize this interaction. To calculate binding affinities between SRSF3 and pri-miR-10b we performed filter binding assays (Figure 3.3). Radiolabeled pri-miR-10b was incubated with increasing amounts of either rSRSF1 or rSRSF3. We observe that rSRSF1 binds pri-

miR-10b at a relative K_D of 21nM +/- 2 while SRSF3 binds at a relative K_D of 82nM +/- 9 (Figure 3.3). Interestingly the Hill coefficient for SRSF1 and SRSF3 are 1.9 +/- 0.4 and 3.1 +/- 1.3, respectively, suggesting that despite having a weaker affinity, SRSF3 may bind cooperatively to pri-miR-10b (Figure 3.3).

SRSF1 and SRSF3 coregulate mature miR-10b activity

We next wanted to examine if SRSF1 and SRSF3 can enhance mature miRNA activity *in vivo* in a coordinated manner. To do so SRSF1 and SRSF3 were co-overexpressed at varying titrating concentrations in HEK293T cells and measured miR-10b activity as fold repression. In the lack of exogenous SR protein, repression of the reporter is lowest. We observe that when SRSF1 and SRSF3 levels are both 100% (5ug) that miRNA repression under each condition is ~2 fold, indicating that SRSF1 and SRSF3 are positively influencing processing (Figure 3.4A). Interestingly, when SRSF1 and SRSF3 are coexpressed at an equal ratio (2.5ug each), or 1:3 ratio (1.25ug:4ug), we observe slightly higher repression at ~2.4 fold (Figure 3.4A). This observation is significant when comparing SRSF1 only overexpression to 1:3 SRSF1:SRSF3 co-expression, suggesting that SRSF3 might enhance SRSF1's effect on miR-10b biogenesis.

Furthermore, we wanted to see if cotransfection with exogenous pri-miR-10b would further enhance the observed results, as endogenous miRNA levels might result in only subtle changes. To do so we co-expressed a pri-miR-10b construct along with SRSF1 and SRSF3, and the miR-10b reporter. As expected we observe an increase in fold repression with the expression of exogenous pri-miR-10b compared to just endogenous pri-miRNA expression (Figure 3.4B). Taken together, SRSF1 and SRSF,

in concert, can enhance mature miRNA activity.

Structural context CNNC motif

Sequence and structural motifs have been shown as determinants of miRNA processing (Du et al. 2015; Auyeung et al. 2013). SRSF3 binding has been shown to have preference for a downstream CNNC motif. Recently it was discovered that RNA structure can affect the accessibility of the CNNC (Fernandez et al. 2017). Thus we hypothesize the presence of structured elements within the SR protein binding site flanking the pri-miRNA hairpin.

To test our hypothesis that SR proteins bind to secondary structured regions we used a selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE). SHAPE allows us to identify secondary structures of RNA by chemical modification of nucleotides. 1M7 (1-methyl-7-nitroisatoic anhydride) acetylates single-stranded, exposed nucleotides. Upon reverse transcription (RT) by primer extension the modified nucleotides result in truncated cDNA. Capillary electrophoresis reveals the RT stops and secondary structure can be derived. SHAPE is a straightforward yet powerful tool to examine secondary RNA structures *in vitro*.

We chose our candidate miRNAs to reflect potential differences in structure based on if they are bound by SR proteins and the presence of a CNNC motif downstream from the hairpin. Our candidate pri-miRNAs include pri-miRs-7-1 and -100. Pri-miR-7-1 contains a CNNC motif, and is bound by both SR proteins. Pri-miR-100 is bound sloley by SRSF3 and contains a CNNC motif.

When examining the secondary structure of the candidate miRNAs there are some interesting elements present in each. Firstly a high confidence pri-miRNA hairpin, with a reactive apical loop, is observed for both pri-miRNAs (Figure 3.5). Previously generated CLIP data sets have shown pri-miR-7 to be bound by both SRSF1 and SRSF3 (Dargyte et al. n.d. and Figure 3.1). Upstream of the hairpin from 38-57 nts is a highly reactive region likely to be single-stranded. Immediately downstream of the hairpin (161-170 nts) is non-reactive and the CNNC containing regions appears to be a structured, double stranded segment (Figure 3.5A). Pri-miR-100 is predicted to be bound only by SRSF3. Both up and downstream regions are generally unstructured, with some short high confidence double stranded regions. Interestingly we observe 6-8nt long bulges at the lower portion of the hairpin (Figure 3.5B).

Overall, pri-miR-7 has the most structured flanking segments relative to the hairpin, which might be reflective of regions for SRSF1 and SRSF3 interactions. While the flanking regions surrounding the pri-miR-100 hairpin are relatively unstructured. Suggestive of structure independent protein-RNA interactions.

SR proteins are predicted to interact with core and auxiliary proteins involved in miRNA biogenesis

We further wanted to identify any possible interactions between SRSF1 and SRSF3 and critical miRNA biogenesis proteins. Using BioGrid we pulled out observed protein interactors for SRSF1, SRSF3, Drosha, DGCR8, and Dicer. Overlapping protein interactions can be seen in Table 3.2, organized by how many times an interaction is observed. SR proteins and Microprocessor components interact with 16 unique proteins. Interacting proteins include: SR domain kinases, splicing factors, and heli-

cases. Two proteins that have been previously uncharacterized as miRNA regulators that emerged in this data set are, CUL3 and ESR1. CUL3 is a conserved protein and a component of a scaffolding complex for E3 ubiquitin ligases (Wimuttisuk et al. 2014). While ESR1 is an estrogen receptor protein. Although not studied together as a whole, SR proteins, and the aforementioned miRNA biogenesis associated proteins might be working in concert to regulate and guide pri- and pre- miRNAs during biogenesis. It would be of interest to explore how these proteins interact with miRNAs and if changes in their expression can alter miRNA levels or activity.

Discussion

The SR protein functional repertoire has vastly expanded since their discovery. Their main roles in regulation of protein coding have long been studied, recently an understanding of how they regulate noncoding RNAs has emerged. In this study we expand upon how SRSF1 and SRSF3 regulate miRNA biogenesis. By using previously published CLIP datasets we further characterize a global interaction between SRSF1 and SRSF3 and pri-miRNAs. We also observe a novel interaction between SRSF3 and mature miRNA sequences in HeLa cells. We characterize *in vitro* binding affinities for SRSF1/SRSF3 for pri-miR-10b. Furthermore, we show that SRSF1 in combination with SRSF3 can stimulate miRNA biogenesis of miR-10b *in vivo*. We believe this paper further expands the range of SR protein post-transcriptional gene regulation.

Similar to Du et al, we hypothesize a processing step prior to Drosha cleavage. We believe this regulatory step is mediated by interactions between RBPs and *cis*-elements outside of the pri-miRNA hairpin. During splicing, SR proteins coordinate exon definition through protein-protein and protein-RNA interactions which allows

for spliceosome formation (Pandit et al. 2013; Howard et al. 2018). Similarly, SR proteins can coordinate binding surrounding the miRNA hairpin, defining the region for processing by the Microprocessor complex. It is understood that pri-miRNAs must contain single-stranded regions up and downstream of the hairpin for efficient Drosha cleavage; our SHAPE data reveals that this region can be double stranded *in vitro* (Ma et al. 2013). We hypothesize that perhaps SR proteins bind to sequences outside of the hairpin and promote their single-strandedness allowing for Microprocessor binding and ultimately Drosha cleavage. SR proteins might bind to nascent pri-miRNAs during transcription to stabilize the strand, preventing R-loops and exposing the double stranded hairpin allowing for Drosha binding.

SRSF1 and SRSF3 have previously been shown to not interact with the Microprocessor, suggesting they bind before the Microprocessor, or form an auxiliary complex. We examined what protein interactions occur between major microRNA biogenesis proteins, SRSF1, and SRSF3 (Table 3.2). Interestingly we notice all have an overlap with two proteins, ESR1, a nuclear estrogen receptor transcription factor and CUL3, a conserved scaffolding protein for E3 ubiquitin ligase. CUL3 might be influencing miRNAs processing by regulating proteins involved in the biogenesis pathway. Despite CUL3 being implicated in mature miRNA degradation little is known as to how it might regulate pri- or pre- miRNAs directly (Han et al. 2020). Although SR proteins and miRNAs are dysregulated the interaction between ESR1 and aforementioned proteins has been observationally characterized. ESR1 positive (ER+) cancer cells result in decreased Drosha expression, which is interesting as ER+ breast cancer tissue and cells have a global increase in miRNA levels (Macias, Michlewski, and Cáceres 2009; Blenkiron et al. 2007). Additionally, ER- cells have increased levels of Dicer and Dicer binding partner TRBP, while Ago2 levels are decreased (Cheng et

al. 2009). This suggests despite increased levels of miRNAs they are not loaded into RISC and not able to target overexpressed cancer genes. It is unclear how SR proteins and ESR1 co-regulate miRNA biogenesis proteins; there might be an unidentified axis between the three.

We observe that SR proteins and DGCR8 can both bind DHX9, DDX17 helicases. Helicases are often in complex with both the Microprocessor in the nuclease and Dicer in the cytoplasm (Kawai and Amano 2012; Robb and Rana 2007). Helicases can unwind the stem of pri- and pre- miRNAs altering accessibility and stability of the transcripts. It is unclear whether DHX9 or DDX17 specifically are unwinding bound transcripts. It is interesting that SRSF1, which has been shown to contain annealing properties, is observed to be interacting with the helicases DHX9 and DDX17 (Table 3.2) (Krainer, Conway, and Kozak 1990). Perhaps these proteins work in maintaining stable structures during the first cleavage step by Drosha.

Some important factors to consider for future studies include, examining what post-translational modifications might be regulating SR proteins while interacting with pri-miRNAs. SR protein RS domains can be heavily phosphorylated resulting in different structural confirmations, protein interactions, and localization (Gui, Lane, and Fu 1994; Xiao and Manley 1997; Cho et al. 2011; Xiang et al. 2013). Insight into the phosphorylative state can define if pri-miRNAs are sequestered to subcellular localization, or transported. As the Microprocessor and SR proteins do not directly interact it would be of interest to also determine if SR proteins are binding miRNAs alone, or as part of a larger complex. This would also explain any disparities observed between *in vivo* and *in vitro* experiments. Our *in vitro* experiments utilize recombinant purified SR proteins rather than a natural cellular complex. Finally, it is of inter-

est to explore if SR protein binding causes nucleotide positional shifting of Drosha cleavage causing a slightly shifted pre-miRNA or mature miRNA sequence, and if these transcripts are productive. Great progress in understanding a mechanism for SR proteins since the first paper explored this novel interaction, more work is needed to define context and physiological consequences.

Methods

Cell Culture

Hek293T cells were grown in DMEM (Signa) supplemented with 10% FBS. At 70% confluence cells were transfected with plasmids using Lipofectamine 2000 protocol (ThermoFisher).

Structure probing

1.2 pmols of RNA in 50 mM Na-HEPES (pH 8) was denatured at 95ºC for 3 minutes, after cooling to room temperature, 10 mM of MgCl2 was added to the solution. RNA was modified in presence of 5 mM of 1-methyl-7-nitroisatoic anhydride (1M7, provided by Dr. Manuel Ares) at room temperature for 15 minutes, followed by quenching with 2.55 M Na-MES (pH 6) for 10 minutes. RNA was washed with 70% ethanol and purified with AMPure Beads (Beckman Coulter). After elution in water, RNA was reverse transcribed using standard SuperScript III protocol (ThermoFisher) and hydrolyzed by 200 mM NaOH. cDNA is purified as previously described on AMPure beads and eluted with 11ul of formamide-ROX350 mix (ThermoFisher) for 15 minutes at room temperature. Primer extension products were analyzed by Elim

Biopharmaceuticals by capillary electrophoresis. Data from capillary electrophoresis runs was analyzed with HiTRACE MATLAB package as previously described (Yoon et al. 2011; Palka et al. 2020).

In vitro transcription

RNA was generated either by transcribing a linearized plasmid with T3 RNA polymerase. T7 RNA polymerase templates were generated using the primerize protocol, where oligonucleotide sequences were PCR amplified using Phusion polymerase (ThermoFisher). *In vitro* transcription was performed using T3 or T7 MegaScript Kit (ThermoFisher). After transcription the RNA was heat to 90°C for 3 minutes and run on a 6% denaturing gel. RNA was visualized by UV shadowing and ex*cis*ed from the gel with a clean razor blade. RNA was eluted overnight shaking in a buffer containing 0.3 M NaOAc and 3% SDS. After elution RNA was ethanol precipitated, washed, resuspended in RNase free water and stored at -20°C until use.

Filter binding

0.25 nM of body labeled RNA was folded for 3 minutes at 90°C in a buffer of 50 mM HEPES (pH 8.0) and cooled to room temperature over 15 minutes. RNA was allowed to bind SRSF1 and SRSF3 for 30 minutes at 37°C after the addition of 10 mM MgCl2. Meanwhile, nitrocellulose (Amersham) and positively charged nylon membrane (Ambion) were pre-soaked in 50 mM HEPES (pH 8.0) and 10 mM MgCl2. Membranes were layered, with nitrocellulose on bottom, in a 96-well vacuum manifold dot blot apparatus (Whatman) and binding reactions were individually pipette into wells. Using a house vacuum samples were filtered through the membranes

and washed with 1 mL pre-soaking buffer. Membranes were air dried and exposed overnight on a phosphor screen, and visualized with a Typhoon image scanner (GE Healthcare). Lane analysis of blots was performed using imageJ, background was determined in the absence of protein. Filter binding data were plotted as fraction RNA bound, using GraphPad Prism to generate Hill coefficients and observed dissociation constants.

Analysis of par-CLIP, and fr-iCLIP datasets

PAR-CLIP data for SRSF3 from HeLa cells was downloaded from (GSM1826788 and GSM1826793). Fr- iCLIP data for SRSF3 from P19 cells was downloaded from (GSE30567). Reference genomes for subsequent analysis were hg19 for HeLa cell PAR-CLIP, and mm9 for P19 fr-iCLIP. Downloaded bed files containing peaks as intervals. Peaks were aggregated to single position binding sites by selecting the center of each interval. Using miRBase 2.0 defined pre-miRNA hairpins as reference, 100 bp were extended on either side of the hairpin. FindOverlaps function from the R package GenomicRanges was used to compute overlaps and relative positions between miRNAs and CLIP sites (Lawrence et al. 2013).

Luciferase assays

MicroRNA reporters using pMIR luciferase reporters were previously generated by Dargyte et al. MicroRNA reporters were co-transfected with TK-rLUC (Promega) as a transfection control. For each 24 well plate 1000 ng of T7-SRSF1 or SRSF3, 200 ng pMIR, 200 ng pGK (exogenous pri-miRNA if used) and 50ng of TK-rLUC we transfected using Lipofectamine 2000 (ThermoFisher). Titration experiments use above

mentioned concentrations of either T7-SRSF1 and/or T7-SRSF3. Activity was assayed 24 hours post transfection using Dual-Glo Luciferase Assay System (Promega). Western blots were performed to confirm SR protein overexpression.

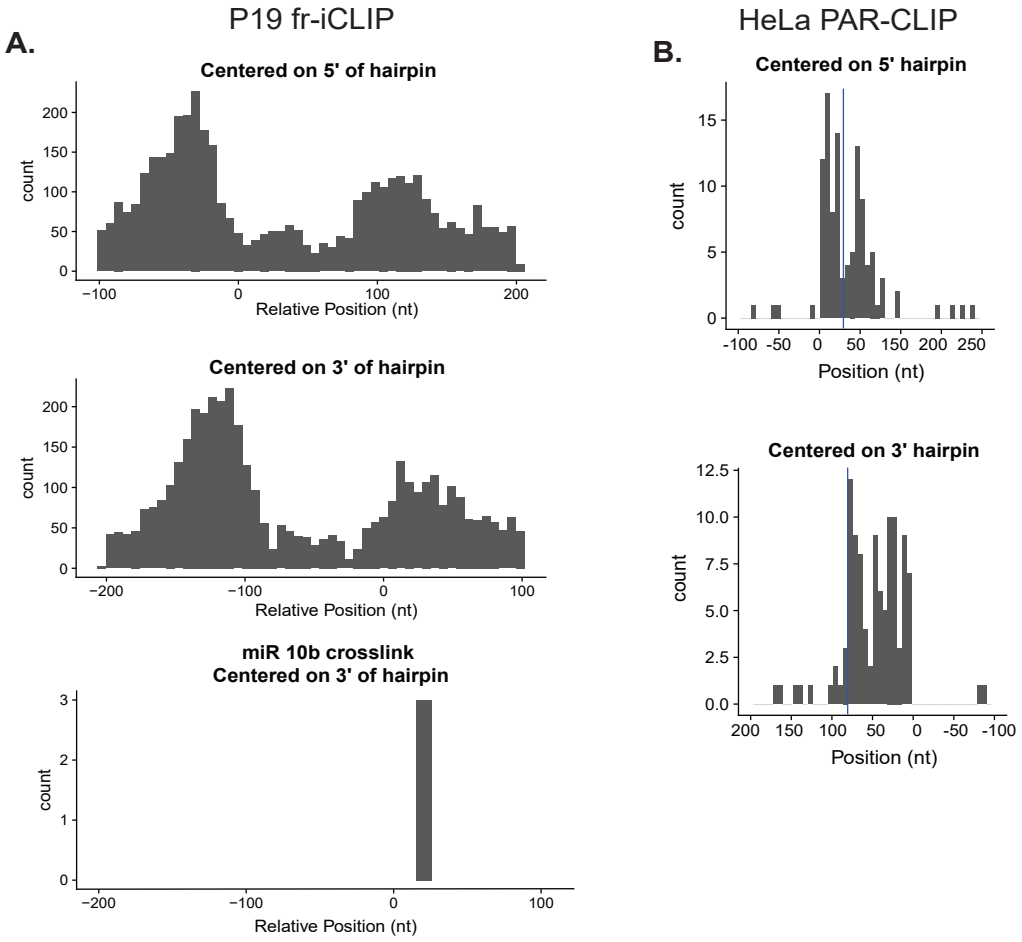


Figure 3.1. Global interactions of RNA binding proteins in different cell types. (A) SRSF3 PAR-CLIP from HeLa cells. PAR-CLIP sequencing counts relative to the 5' or 3' of annotated pri-miRNA hairpins. 5' and 3' hairpin boundaries annotated as 0. The blue line denotes the position where SRSF3 binds miR-10b relative to miR-10b hairpin 5'/3' ends. (B) SRSF3 fr-iCLIP in mouse P19 cells. Similar to before, SRSF3 iCLIP sequencing counts were plot relative to 5' and 3' of pri-miRNA hairpin. SRSF3 crosslink to miR-10b is also observed downstream of the hairpin. Note the differences in SRSF3 crosslinking comparing human and mouse CLIPs.

not aligned with mature miRNA	aligned with mature miRNA
hsa-mir-30e	hsa-mir-200b
hsa-mir-30c-1	hsa-mir-101-1
hsa-mir-92b	hsa-mir-320b-1
hsa-mir-205	hsa-mir-181a-1
hsa-mir-1246	hsa-mir-320b-2
hsa-mir-146a	hsa-mir-128-1
hsa-mir-340	hsa-mir-10b
hsa-mir-148a	hsa-mir-26b
hsa-mir-106b	hsa-mir-26a-1
hsa-mir-30d	hsa-mir-128b
hsa-mir-23b	hsa-mir-143
hsa-mir-27b	hsa-mir-218-2
hsa-mir-532	hsa-mir-30a
hsa-mir-125b-1	hsa-mir-25
hsa-mir-3613	hsa-mir-182
hsa-mir-342	hsa-mir-183
hsa-mir-629	hsa-mir-29a
hsa-mir-328	hsa-mir-320a
hsa-mir-301a	hsa-mir-101-2
hsa-mir-769	hsa-mir-7-1
hsa-mir-125a	hsa-mir-24-1
hsa-mir-125b-2	hsa-mir-181a-2
hsa-mir-155	hsa-mir-199b
hsa-mir-185	hsa-mir-221
has-let-7g	hsa-mir-500a
has-let-7i	hsa-mir-502
	hsa-mir-92a-2
	hsa-mir-224
	hsa-mir-452
	hsa-mir-146b
	hsa-mir-100
	hsa-mir-200c
	hsa-mir-26a-2
	hsa-mir-92a-1
	hsa-mir-203a
	hsa-mir-7-2
	hsa-mir-22
	hsa-mir-423
	hsa-mir-10a
	hsa-mir-142
	hsa-mir-21
	hsa-mir-122
	hsa-mir-7-3
	hsa-mir-24-2
	hsa-mir-99a
	has-let-7a-1
	has-let-7-1
	has-let-7d
	has-let-f-2
	has-let-7a-2
	has-let-7c
	has-let-7a-3
	has-let-7b

Table 3.1. SRSF3 associated miRNAs defined by PAR-CLIP in HeLa cells.

MiRNAs associated with SRSF3 CLIP sequencing tags. MiRNAs were examined if previous experiments identified the mature miRNA sequence and its expression. SRSF3 CLIP tags were grouped based on if they overlap with known mature miRNA

sequences in the UCSC human genome browser (hg19). SRSF3 crosslink tags that do not overlap with a miRNA cannot be distinguished between if the mature sequence has not been annotated or if SRSF3 is binding to a different region altogether.

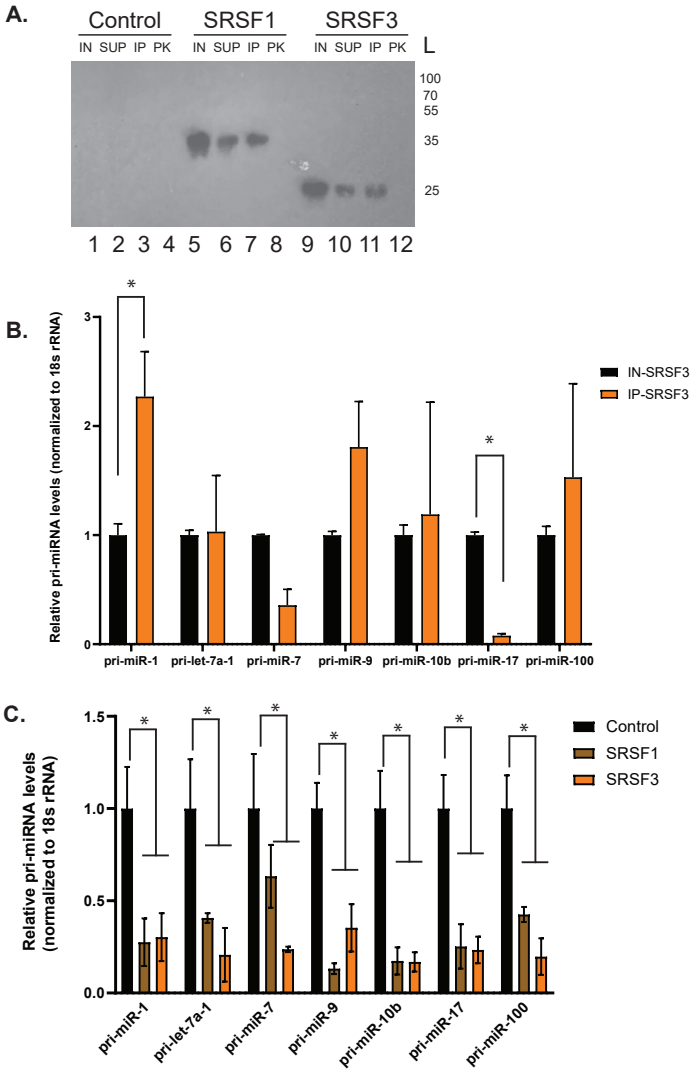
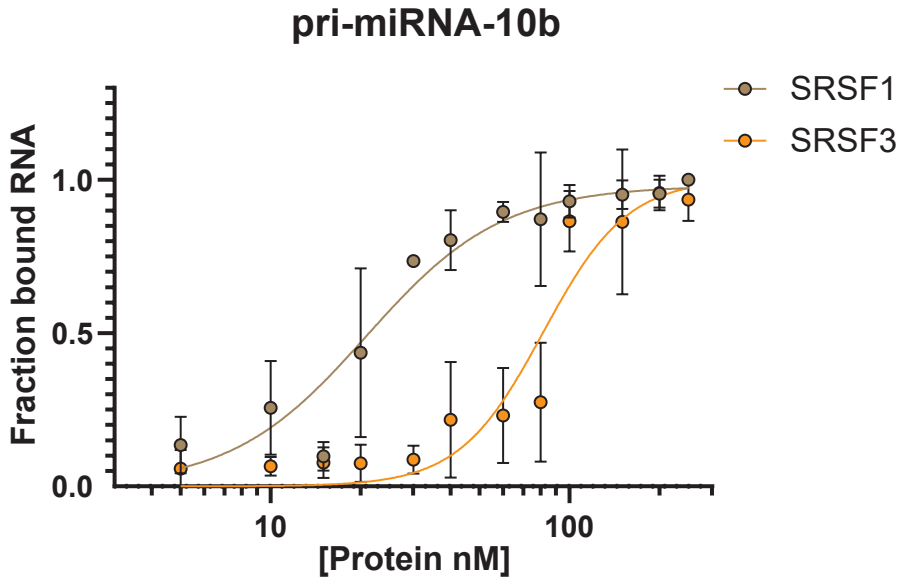


Figure 3.2. SRSF1 and SRSF3 bind pri-miRNAs and alter pri-miRNA steady state levels ***in vivo*****.**

(A) Anti-T7 western blot of samples collected from T7- RNA immunoprecipitation. Samples were from HEK293T cells overexpressing control, T7-SRSF1, or T7-SRSF3 plasmids. For each condition input lysate (IN), post-immunoprecipitation supernatant (SUP), immunoprecipitated sample (IP), and proteinase K treated IP sample (PK) were collected. (B) RT-qPCR of T7-RIP for cells over expressing control, SRSF1,

and SRSF3 plasmids. Samples were normalized to an endogenous gene, 18s rRNA, as well as respective inputs. Significance was determined by unpaired T.test, * < 0.05. (C) RT-qPCR of input lysate samples from (A), for control, SRSF1, and SRSF3 over-expression. Different pri-miRNA levels were measured and normalized relative to an endogenous gene and control input samples.

A.



B.

	Dissociation constant (KD)	Hill coefficent
SRSF1	21 +/- 2	1.9 +/- 0.4
SRSF3	82 +/- 9	3.1 +/- 1.3

Figure 3.3. SRSF1 and SRSF3 interact with pri-miR-10b *in vitro.*

(A) UV label transfer and western blot of SR proteins. Not the presence of high molecular weight bands in absence of RNase, in presence of SR proteins. T7 western blot confirms the presence of SR proteins in presence or absence of RNase. (B) *In vitro* filter binding assay measures observed affinity of SRSF1 and SRSF3 for pri-miR-10b. (C) Calculated dissociation constants and hill coefficients using filter binding assay (B) for SRSF1 and SRSF3.

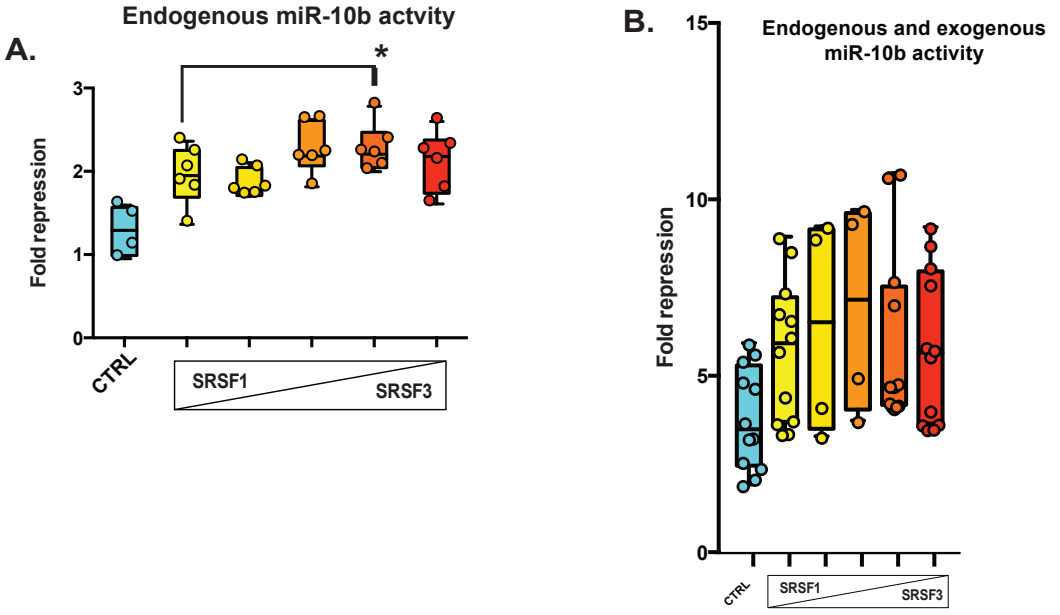


Figure 3.4. SRSF1 and SRSF3 coregulate pri-miR-10b processing *in vivo*

(A,B) Luciferase reporter activity of pMIR-10b with exogenous overexpression of pri-miR-10b (A) Endogenous miR-10b activity measured by pMIR-10b. Cells were co-transfected with differing ratios of T7-SRSF1, and T7-SRSF3. Significance was determined by paired T.test, * < 0.05. (B) MiR-10b activity measured by pMIR-10b after transfection of exogenous pri-miR-10b along with T7-SRSF1 and T7-SRSF3 at differing ratios. Significance was determined by paired T.test, * < 0.05.

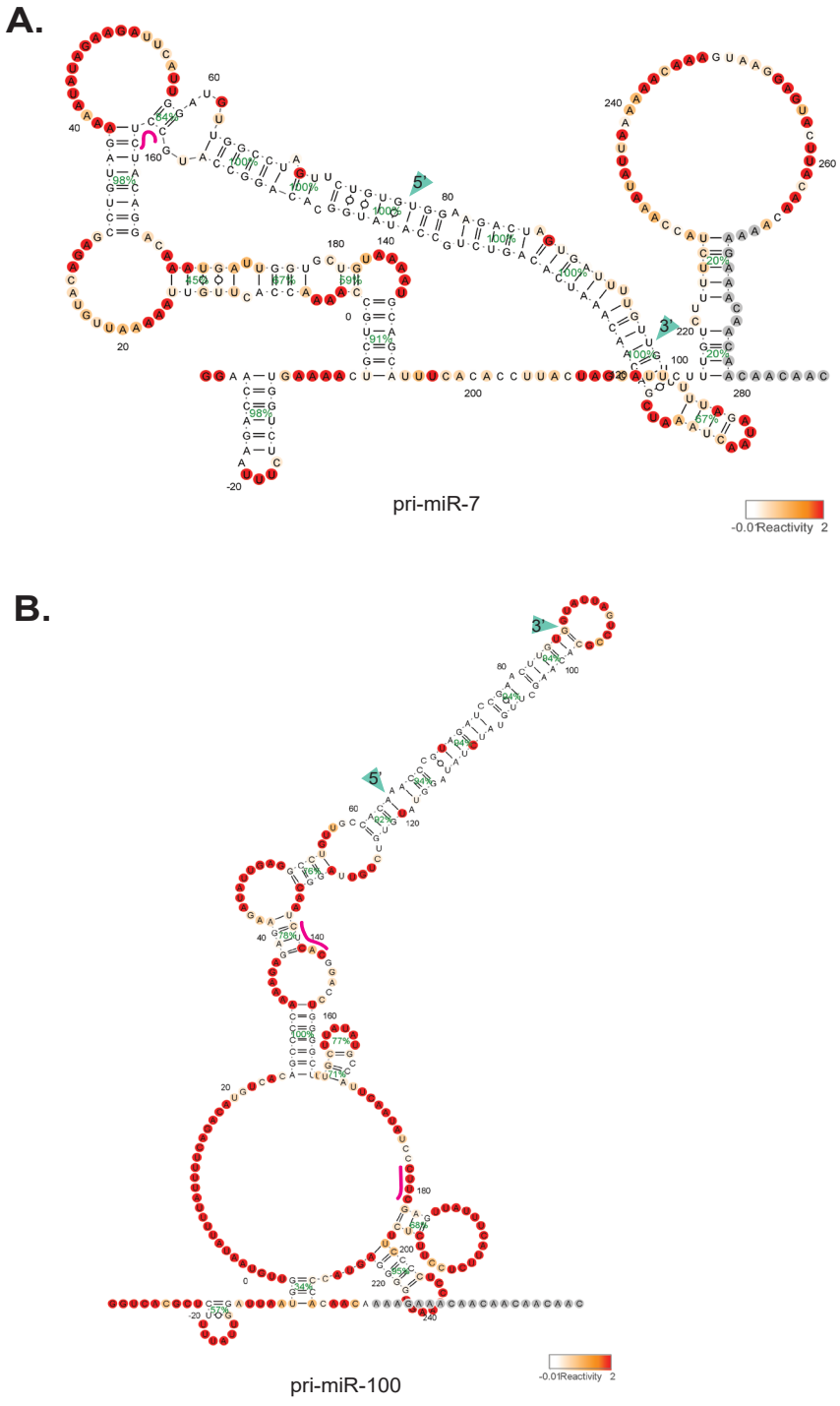


Figure 3.5. Observed secondary structures of pri-miRNAs predicted to be bound by SR proteins

Secondary structures of pri-miR-1 (A), pri-miR-7 (B) pri-miR-100 as determined by SHAPE with 1M7. Reactivities were assigned by Hi-TRACE. Nucleotides highlighted with warmer (red) colors have highest reactivities, indicative of 1M7 modifications. The 5' and 3' ends of the embedded mature miRNA are annotated with green arrows. Potential SRSF3 targeted CNNC motifs are marked with a pink line pri-miR-7 (156-159nt) and pri-miR-100 (137-140 & 178-181nts).

DROSHA	DGCR8	DICER
CUL3	CUL3	CUL3
ESR1	ESR1	ESR1
SRPK1	SRPK1	
SRPK2	SRPK2	
WWP2	WWP2	
	DHX9	DHX9
	DDX17	
	STAU1	STAU1
KIF23	APP	PLEKHA4
RBM39	EWSR1	RECQL4
	FUS	SYNCRIP
	HNRNPU	
	SNRNP70	

Table 3.2. Predicted interactions between miRNA biogenesis associated proteins and SR proteins

Interactors overlap between SRSF1, SRSF3 and Drosha, DGCR8 or Dicer. Interactions were derived from BioGrid. List is grouped by recurring interactions between groups.

Chapter 4: Conclusions and future directions

In this thesis, I have presented research on how two RNA binding proteins, SRSF1 and SRSF3 can regulate microRNA biogenesis. Using miR-10b as our model, I define SRSF1 as binding a single-stranded region upstream of the pri-miRNA hairpin. Using both *in vivo* and *in vitro* experiments I was able to characterize SRSF1-dependent regulation to positively influence miR-10b processing. Furthermore, I have begun to explore the co-regulation of SR proteins during miRNA biogenesis. SRSF3 has been previously characterized in promoting the biogenesis of pri-miRNAs by binding to a motif downstream of the pri-miRNA hairpin. Using *in vivo* miRNA reporters, I observe that co-overexpression of SRFS1 and SRSF3 can result in significant increase in mature miR-10b activity. Knowing that SR proteins coordinate interactions during pre-mRNA splicing. I hypothesize that SR proteins coordinate binding to flanking regions outside of the hairpin, promoting a more accessible secondary structure of the hairpin which allows for recruitment of the Microprocessor complex during the initial steps of miRNA processing.

I believe I have contributed to previous studies but also expanded the role for SR proteins during miRNA biogenesis by interacting with sequences upstream of the pri-miRNA hairpin. There is still much to learn about how SR proteins regulate microRNA biogenesis. I began interrogation of the understudied region upstream of the pri-miRNA hairpin. I believe future experiments should focus on the biochemical and *in vivo* interaction between the SR protein and pri-miRNAs in this region. One immediate goal is to optimize structure probing of RNAs in the presence of recombinant protein to observe any changes in structure during and after protein binding. From a cellular and molecular aspect, we can examine subcellular localization of SR-miRNA

complexes, and characterize the domain requirements of SR proteins interacting with the RNA. Immunoprecipitation and mass spectrometry based experiments may reveal an SR protein containing complex prior to Microprocessor binding that regulates biogenesis. I would also like to characterize the sequence context of the pri-miRNA bound by SR proteins, is it a full length pri-miRNA or a truncated transcript similar to pro-miRNAs. Lastly, it would be interesting to characterize the cleavage site produced by the Microprocessor in the presence of SR proteins. SR proteins might be directing Microprocessor cleavage of the hairpin, contributing to production of efficient pre-miRNAs.

I am optimistic that my thesis will further the understanding of SR dependent regulation of early miRNA biogenesis.

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