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SANTA CRUZ

BINDING INTERACTIONS BETWEEN SPLICING FACTOR CUS2 AND U2 snRNA

A thesis submitted in partial satisfaction
of the requirements for the degree of

MASTER OF ARTS

in

BIOLOGY

by

Santiago Sanchez

June 2019

The Thesis of Santiago Sanchez is approved:

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BINDING INTERACTIONS BETWEEN SPLICING FACTOR CUS2 AND U2 snRNA

SANTIAGO SANCHEZ

ABSTRACT

Assembly of the U2 small nuclear ribonucleoprotein (snRNP) to form the pre-spliceosome (PSP) is the first ATP dependent step of splicing (Kim & Lin 1993). This assembly requires the rearrangement of the U2 stem II region from a stem IIc conformation to its competing stem IIa conformation (Zavanelli & Ares 1991, Perriman & Ares 2000). Both smFRET and genetic studies have shown splicing factor Cus2 is able to influence refolding of U2 into its IIa conformation. (Yan 1998, Perriman & Ares 2007, Rodgers et al. 2016).

The *Saccharomyces cerevisiae* splicing factor CUS2 (Cold sensitive U2 snRNA Suppressor 2), contains an RNA recognition motif (RRM) which binds to U2 snRNA, as well as an acidic C-terminus which contains amino acid residues involved in Cus2's RNA refolding and its cold sensitivity suppression. A Cus2-Y48D mutant

(hereafter referred to as Y48D) reduces the protein's ability to bind U2 RNA in vitro, and the proteins ability to suppress cold sensitivity seen in stem IIa folding mutants (Yan et al. 1998, Rodgers et al. 2016). Additionally, the mutations in Cus2's C-terminus enhance its RNA refolding and cold sensitivity suppression, implying that the C-terminus is involved in both processes. Lastly, this enhanced suppression and enhanced refolding is only possible in the absence of the Y48D mutation, suggesting that the C-terminus interacts with CUS2's RRM, though the nature of this interaction remains unclear.

Surprisingly, although the Y48D mutation weakens Cus2's ability to bind wild type U2, base pairing mutations that stabilize U2 in the IIc form allow this same mutant to bind the snRNA through a novel interaction (Rodgers et al. 2016). This interaction, along with knowledge that Cus2 preferentially binds to IIc-U2 over IIa (Perriman & Ares 2007), presents a model in which a novel RNA binding site outside Cus2's RRM binds specifically to stem IIc U2, and then hands off the refolded stem IIa to the canonical RRM. Furthermore, it seems possible that the C-terminus may be interacting with this putative binding site or the canonical RRM to possibly inhibit RNA binding.

Here I have tested parts of this model using genetic and biochemical approaches. To observe the effects of a hypothetical second binding site, I tested for predicted growth defects in yeast expressing only a stem IIc stable U2 and the Y48D binding mutant protein. My results suggest that the combination of a IIc stable U2 and the Y48D protein does not cause a significant growth defect, and that this binding

interaction might not be strong enough to halt U2 snRNA refolding, and inhibit splicing progression.

To further explore the binding interaction between Cus2 and U2 I tested the affinity between different U2 stem II mutants and Cus2 or the Y48D mutant protein. If Cus2 protein does contain two RNA binding sites that discriminate between either conformation of stem II, it could be that the mechanism behind refolding utilizes different affinities for U2 stem IIa and IIc at each site. I therefore measured the binding affinity of Cus2 and Cus2-Y48D to U2 stem II stabilized in either its IIa or IIc form. My data indicates that the Cus2 protein binds to U2 snRNA in a fashion largely dependent on the presence of its RRM and access of the RRM to its target.

Acknowledgements

I'd like to thank my advisor Manny Ares for essential insights and advice regarding this project. I'd like to thank members of my thesis committee: John Tamkun for insights into the importance of my work and Melissa Jurica for support and theoretical discussions. I'd also like to thank my family, and my partner Ashley Bratrude for encouragement and unwavering support.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Rearrangement of U2 snRNA and pre-spliceosome assembly

Splicing is the processing of pre-messenger RNA (pre-mRNA) into mature mRNA molecules and is vital to correct gene processing in all eukaryotic cells. The splicing machinery (the spliceosome) is composed of five small nuclear ribonucleic acids (snRNAs) and a multitude of splicing factors, which carefully position the snRNAs around the pre-mRNA being processed (Fica et al. 2017). Together, snRNAs and their associated proteins are called small nuclear Ribonuclear Protein particles (snRNPs). As the spliceosome operates, these snRNPs undergo drastic structural rearrangements in the form of dynamic RNA-RNA, RNA-protein and protein-protein interactions (Fica et al. 2017). One such rearrangement takes place just prior to the first ATP dependent step of splicing: stable binding of the U2 snRNP to the intron branch point, forming the pre-spliceosome (PSP).

U2 snRNA contains an essential stem-loop which can adopt two mutually exclusive competing structures: stem IIa and stem IIc (**Figure 1**) (Ares & Igel 1990). During the first catalytic step of splicing, U2 snRNA's stem II rearranges into its IIc form, and before the U2 snRNP can assemble for another cycle of splicing on a new intron, stem II must refold back into its IIa conformation (Perriman & Ares 2007, Hilliker et al. 2007, Fica et al. 2017). Mutations to U2 snRNA that disrupt stem IIa or stabilize the competing stem IIc conformation, such as G53A, inhibit PSP formation and cause a cold-sensitive phenotype in yeast cells (Ares & Igel 1990, Zavanelli &

Ares 1991, Yan et al. 1998, Rodgers et al. 2016). The cold sensitive G53A U2 snRNA mutation inhibits the refolding of U2 snRNA from its stem IIc to its stem IIa form by destabilizing the end of the stem IIa helix, disallowing the spontaneous interconversion between the two conformations. In such a case, the inability of U2 to adopt the IIa conformation thermodynamically causes an inability to carry out splicing, and hence a decrease in cell growth.

U2 snRNA conformation is influenced by the Cus2 protein

In an attempt to find factors that influence U2 snRNA structure, a screen for suppressors of cold sensitivity caused by a G53A U2 snRNA mutation yielded the CUS1, CUS2, and CUS3 genes (Wells et al. 1996). The CUS2 gene is non-essential in *Saccharomyces cerevisiae*, yet it is highly conserved among eukaryotes (**Figure 2**) (Yan et al. 1998). The Cus2 protein contains an RNA recognition motif (RRM) which contains a tyrosine (Y48) necessary for both its RNA binding and for its cold-sensitivity suppression in vivo, arguing that RNA binding is essential for its activity in the cell (Yan et al. 1998). Cus2 also contains a U2 association factor Homology Motif (UHM) responsible for binding the UHM Ligand Motif (ULM) of Hsh155 (Talkish et al. 2019). Once bound, Cus2 must be removed by the ATPase activity of Prp5 in order for pre-spliceosome assembly to progress (Perriman & Ares 2007). The chronological order behind Prp5's ATP-ase activity, removal of Cus2 from Hsh155, and Cus2's stem-II refolding activity is unknown. Lastly, the protein's acidic C-

terminus holds two residues which when mutated (D282N, and L284F) enhance the protein's ability to refold U2 into its IIa form (Rodgers et al. 2016).

Model behind CUS2's RNA refolding mechanism

Although the two conformations of U2 snRNA exist in dynamic equilibrium, Single molecule fluorescence resonance energy transfer (smFRET) data shows Cus2 is able to shift the equilibrium of U2 towards its stem IIa conformation (Rodgers et al. 2016). Interestingly, Rodgers et al. (2016) also showed that disrupting Cus2's RRM with a Y48D mutation weakens its ability to bind wild type U2 snRNA, but causes a novel binding interaction with a stem IIc stable U2 snRNA. This data strongly suggests the existence of a second site on Cus2 that binds RNA, and specifically recognizes the IIc form of U2 snRNA.

CUS2 mutations D282N and L284F enhance its ability to both refold U2 snRNA into its stem IIa form and to suppress the cold sensitivity caused by a U2 snRNA mutation. However this enhanced activity is only seen in the absence of the Y48D mutation. Though this connection between the RRM and the C-terminus is striking, the mechanism linking cold sensitivity to Cus2's C-terminal suppressor mutations is still unclear. One possible mechanism involves Cus2's C-terminus blocking its own putative binding site, preventing it from binding to the stem IIc form U2. In such a mechanism the dominant mutations D282N and L284F destabilize the interaction between the C-terminus and the putative RNA binding motif, opening the RNA binding site and hence increasing contact between Cus2 and stem IIc.

Altogether, this data has yielded a model in which Cus2 protein contains two different RNA binding sites which differ in RNA binding specificity (**Figure 3**). One site recognizes U2 snRNA in its stem IIc form, and holds the RNA until either the Cus2 protein has actively folded it into its stem IIa form, or until U2 adopts the IIa form thermodynamically. Then the IIa conformation is recognized by Cus2's canonical RRM, binds to it, and the protein releases IIa form U2 either through a weak binding interaction, or the binding is displaced through the role of Prp5's ATP-hydrolysis.

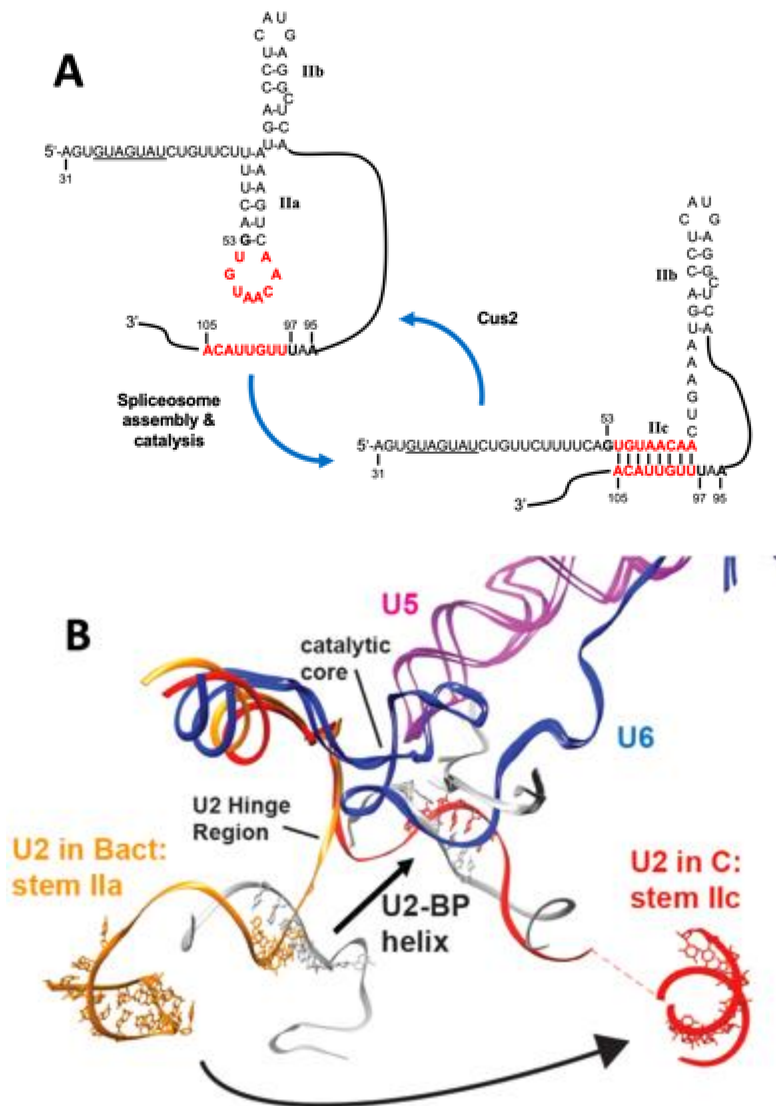

Figure 1: U2 stem loop II rearrangements: **A**) U2 snRNA nucleotides 31-84 and 95-105 from *Saccharomyces cerevisiae* showing stem II in either the IIa (left) or IIc (right) conformations. As the first catalytic step of splicing occurs, U2 snRNA folds into the stem IIc conformation (Ares & Igel 1990, Perriman & Ares 2007, Hilliker et al. 2007). For splicing to continue, U2 snRNA must refold back into its stem IIa conformation. Cus2 protein facilitates the refolding of U2 from its IIc to IIa form. In bold are G53, A95, and U97, which can be mutated to stabilize the IIc form of U2. **B**) Overlap of two Cryo-EM structures of stem II of U2 snRNA in the Bact (orange) or C (red) complexes. Shown are U5 (purple), U6 (blue), the catalytic core, the U2 hinge region, and the U2-branch point helix (U2-BP). As splicing catalysis occurs, the stem II region of U2 changes conformation as a result (Yan et al. 2016, Wan et al. 2016).

```
cerevisiae    ------------------------------------------------------------ 0
pombe         ------------------------------------------------------------ 0
human         ------------------------------------------------------------ 0
worm          -----------------------------------MSDFVPETEENEEE-----SQKIDE 20
fly           MSDEGGCKSEQLEKSEEAEEKKGDAEGQEAKAPILNPISVPEVDDKPTENKPQSDNHADK 60


cerevisiae    ------------------------------------------------------------ 0
pombe         ------------------------------------------------------------ 0
human         ------------------------------------------------------------ 0
worm          IHEIPAQAIIIDQSFSRRFLNNKWYGKYGEDGDLL------------EHIDEDWEPVGLD 68
fly           TDETPSQD----------FAAYEEHMTYGADGGAIYTDPSTKQKYKWCATGNNWQPLGVD 110


cerevisiae    ------------------------------------------------------------ 0
pombe         ------------------------------------------------------------ 0
human         -------------------MSGTNLDGN---------DEFDEQL--RMQELYGDGKDGD 29
worm          EVE----------------FLSQLWFEQEDQEKKAHRHYDWDEEKKEWVPKAKQE----- 107
fly           EDVDGQAEDPYENEHYKWCPKSQQWLPKK--QETETEHYKWDDEQKKWVPKHPNPGQEGV 168


cerevisiae    ------------------------------------------------------------ 0
pombe         ----------------MSSQPFWDERIHRWRCLGSEGNELIYIDEEQTWKDYDPNSLKM- 43
human         TQTDAGGEPDSLGQQPTDTPYEWDLDKKAWFPKITEDF----I------ATYQANYGFSN 79
worm          -------------------------------EVNEDF----I------AEYQANYGVQY 125
fly           CGVDEHGER--TYTDKDGVEFFWDATKSAWFPKIDDDF----M------ARYQMNYGFID 216


cerevisiae    -----MDADELELKGH--LKKLKKEE-LLRRKQLKESNL--------------------Q 32
pombe         NKAGSTGAEVSDVTAEATEGKESSNGEDRHTKRLYES-------TSAEGYPSGSRNKKSK 96
human         DGASSSTANVEDVHAR--T---AEEP-PQ-----------EKAPEPTDARKK-GEKR--K 119
worm          DDIYKKMDEELQEKAA--KAQKEDEE-KKEKKRKK-------------KVGLGA-GE--D 166
fly           NTSAGEKEKA-EKEAA--EAKRKEEE-LKRMTAEAEAAMSRDNPASSAAVPTGKRKA--Q 270
                      .  :  .         .:                                .
                         Y48                 RRM1
cerevisiae    -KRELEYNNASKNTSIYISGLPTDKTTKEGLTEQFCKYGMIRTN-RDGEPLCKLYVNDKG 90
pombe         SENSEASPAPVINKAVYIQGLPLDVTVD-EIEEVFKKCGVIAKNIDNGTPRIKIYRTEDG 155
human         AESGWFHVEEDRNTNVYVSGLPPDITVD-EFIQLMSKFGIIMRDPQTEEFKVKLYKDNQG 178
worm          AKEGWLDLGD-KVHAVYVSNLPEDITDE-EFQKFMSKCGVIQPDIRTNKPKCKLYREENG 224
fly           EPPKWFEMDPLQNTKVYVSNLPLDITMD-EFADLMGKCGMVMRDPQTQKFKLKLYAEKDG 329
                           :*:..** * *  .   :  . : * *::   :         *:*   ..*

cerevisiae    AFKGDALITYSKEESVTLAIEMMNESIFL---GKQIRVERAQFQNKEGDNMHGKENDLKE 147
pombe         TPKGDALIVFFRSESVELAEQLFDDTEFRYGSGQKMRVQKANIDYKKEKTVNK------D 209
human         NLKGDGLCCYLKRESVELALKLLDEDEIR---GYKLHVEVAKFQLKGEYDAS-------- 227
worm          KLKGDGRCCYIKKESVELACNILDGANLN---GREVKVEEARFEMKGDFDPA-------- 273
fly           QIKGDGLCDYIKVESVNLALKILDEYNLR---GHKIRVQRAQFQMRGEYNPA-------- 378
                ***.    : : *** ** ::::    :     * :::*: *.:: :

cerevisiae    FNGPEPPIKRLK----KAKSEGEGEVIDYN------DDESLAKADRTVIFANVFNIYKSY 197
pombe         VGGALKK-KALR-----LRQQQMQQISSWDDVDEEVDDKRKKRFNKIVVLKHIFTLEELD 263
human         ----KKK-KKC--KDYKKKLSMQQKQLDWRP--ERRAGPSRMRHERVVIIKNMFHPMDFE 278
worm          ----RKR-RKLTAAQKKRYMEQQNKIFEWTP--DKPRNY-RPKSDCTVIVKNLFTQEMMN 325
fly           ----LKP-KRKK-KDKEKLQKMKEKLFDWRP--DKLRGE-RSKNEKTVIIKNLFTPELFE 429
                        :            .   :   .:           .     : :   *:.  ::*
                                              UHM
cerevisiae    -TNDDINDIQEDLLEGCEEIGQVDSISVSP--NKGEATVVFKNNKVALQCCKIMTGRYFD 254
pombe         KTPELLIDLKDDITEEAEKCGRVTNVVLYDKEPDGVVTVRFSNNEEAEACVRLMQGRYFD 323
human         DDPLVLNEIREDLRVECSKFGQIRKLLLFDRHPDGVASVSFRDPEEADYCIQTLDGRWFG 338
worm          KNAALMLDLKEEMTQSCQKYGIVKKVVVYANHPDGVVSVTFPTTEESDMAVKYLHGRVVD 385
fly           KEVELILEYQNNLREECSKCGMVRKVVIYDRHPDGVAQINMASPEEADLVIQMMQGRYFG 489
                    : : ::::    ..: * : .: :       .* . : :    : :     : : ** ..
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(Continued on next page)

```
cerevisiae     GQKLLAFISGDENTSSTSDKNE-DSEVE--------DDLI*------------------- 285
pombe          GRVVEASIYDGKVRFQKSGKHTLDDEEDEEKRLEKFADWLENSN---------------- 367
human          GRQITAQAWDGTTDYQVEE-----TSREREERLRGWEAFLNAPEANRGLRRSDS----VS 389
worm           GRKLTAELWDGRTKFKVEE-----TEEDEEKRRKEYEKYIEGGSSETKEESDDDDDD--E 438
fly            QRQLSAEAWDGKTKYKIEE-----SAVEAHERLSKWDEFLAEEETDKKASEDMKEEDVDS 544
                : : *   ..    . .         :           :

cerevisiae     ------------------------------------------------------------ 285
pombe          ------------------------------------------------------------ 367
human          ASERAGPSRARHFSEHPSTSKMNAQETATGMAFEEPIDEKKFEKTEDGGEFEEGASENNA 449
worm           AEDN-------------------------------------------------------- 442
fly            PENQLLPGDATP------------------------------------------------ 556

cerevisiae     ------------------------------------------------------------ 285
pombe          ------------------------------------------------------------ 367
human          KESSPEKEAEEGCPEKESEEGCPKRGFEGSCSQKESEEGNPVRGSEEDSPKKESKKKTLK 509
worm           ------------------------------------------------------------ 442
fly            ------------------------------------------------------------ 556

cerevisiae     ------------------------------------------------------------ 285
pombe          ------------------------------------------------------------ 367
human          NDCEENGLAKESEDDLNKESEEEVGPTKESEEDDSEKESDEDCSEKQSEDGSEREFEENG 569
worm           ------------------------------------------------------------ 442
fly            ------------------------------------------------------------ 556

cerevisiae     ------------------------------------------------------------ 285
pombe          ------------------------------------------------------------ 367
human          LEKDLDEEGSEKELHENVLDKELEENDSENSEFEDDGSEKVLDEEGSEREFDEDSDEKEE 629
worm           ------------------------------------------------------------ 442
fly            ------------------------------------------------------------ 556

cerevisiae     ------------------------------------------------------------ 285
pombe          ------------------------------------------------------------ 367
human          EEDTYEKVFDDESDEKEDEEYADEKGLEAADKKAEEGDADEKLFEESDDKEDEDADGKEV 689
worm           ------------------------------------------------------------ 442
fly            ------------------------------------------------------------ 556

cerevisiae     ------------------------------------------------------------ 285
pombe          ------------------------------------------------------------ 367
human          EDADEKLFEDDDSNEKLFDEEEDSSEKLFDDSDERGTLGGFGSVEEGPLSTGSSFILSSD 749
worm           ------------------------------------------------------------ 442
fly            ------------------------------------------------------------ 556

cerevisiae     ------        285
pombe          ------        367
human          DDDDDI        755
worm           ------        442
fly            ------        556
```

Figure 2: Alignment of CUS2 orthologs: *Saccharomyces cerevisiae,* (top) followed by orthologues from *S. pombe, H. sapiens, C. elegans, and D. melanogaster*. The **RRM** and **UHM** of Cus2 are shown in bold. Punctuations below columns indicate the following: Asterisks (*) indicate complete conservation, a colon (:) signifies conservation of R group properties, and a period (.) signifies conservation of groups with weakly similar properties.

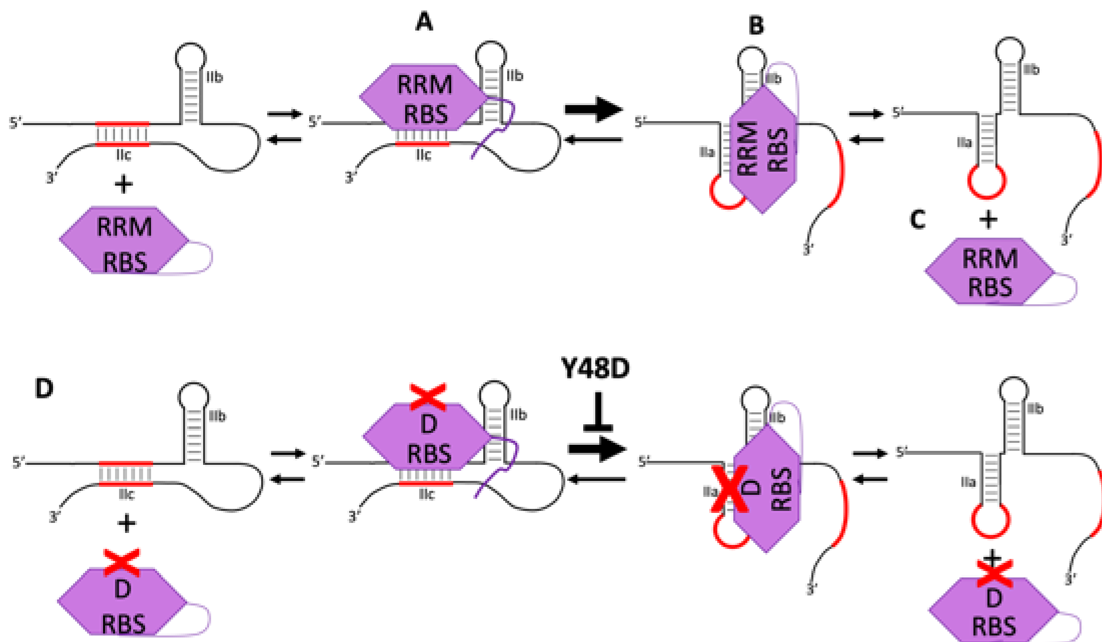

Figure 3: Model for Cus2 refolding mechanism:
A Cus2's putative binding site recognizes stem IIc of U2 snRNA, enabled by displacement of the C-terminal tail.
B Cus2's putative RRM holds U2 until it has refolded to its stem IIa conformation, which is subsequently recognized by the canonical RRM.
C After refolding, Cus2's canonical RRM has a lower (or possibly higher) affinity for stem IIa U2 and Prp5p (not shown) removes Cus2 using its ATPase activity.
D The Y48D mutation, which disrupts Cus2's RRM, is proposed to inhibit binding of U2 in its IIa form, and thereby increasing the binding of Cus2 to the IIc form. This binding might be strong enough to inhibit refolding of the RNA to its IIa form, and sequester the RNA from being assembled into the spliceosome.

CHAPTER 2

MEASURING SPECIFIC Cus2 and U2 snRNA BINDING INTERACTIONS

Thus far, several genetic and physical interactions between CUS2 and other splicing factors have been found. Cus2 interacts genetically with U2 snRNA and with Prp5; CUS2 knockout is synthetic lethal in the presence of U2 folding mutants, and temperature sensitive Prp5 mutants are lethal without CUS2 present (Perriman & Ares 2000). Cus2 also interacts with Prp11, an interaction which is mirrored in human homologs Tat-SF1 and SF3b1 subunit SAP62 respectively. This interaction occurs through the UHM-ULM motifs on the proteins (Yan et al. 1998, Loerch et al. 2018). CUS2's UHM is also responsible for binding to splicing factor HSH155; an interaction which must be disrupted in order for PSP formation to proceed (Talkish et al. 2019). Additionally, Cus2 has been found to coimmunoprecipitate both wild type U2 and IIc-stabilized U2, preferentially binding the IIc form (Perriman & Ares 2007). Yet, despite all these observations, the direct interaction site(s) on Cus2 that bind(s) the competing conformations of U2 snRNP are unknown. Here I aim to elucidate how Cus2 interacts with U2 snRNA by exploring predictions surrounding a hypothesized second RNA binding site on Cus2.

Recombinant Cus2 and Y48D protein expression and purification

To study protein-RNA interactions between Cus2 or its mutant protein Y48D, recombinant proteins were made as follows: Rosetta (DE3) *E.coli* cells were transformed with the pET24 plasmid containing a recombinant construct which contains the T7 promoter, followed by CUS2 or Y48D open reading frames, followed by a C-terminal 6-HIS tag for purification on Nickel ion resin. Two different protein

preps of both wild type Cus2 and mutant Y48D protein were made which differ in the stringency of purification. SS-Cus2 and SS-Y48D protein preps were purified only using a 6-His trap column loaded with $NiCl_2$, and then dialyzed against 200mM NaCl, 25mM HEPES pH 7.4, 3% Glycerol, with added 0.5mM DTT, 0.1mM PMSF, and 0.2mM EDTA. Cal-Cus2 and Cal-Y48D were further purified through a Subtractive Q-HiTrap column and a subsequent Superdex-75 gel filtration column.

Since the 285 amino acid Cus2 protein entirely lacks tryptophan residues and contains only nine phenylalanine residues, the lack of aromatic groups makes measuring the amount of protein using absorbance of 280 nm light unreliable. We therefore measured the concentration of different elution fractions and dilutions of the protein using a Bradford assay and also ran samples on SDS-PAGE protein gels next to BSA Standards to ensure accuracy (**Figure 4**). Furthermore, to ensure that the structural folding of the protein preps was not completely compromised during purification, presence of secondary structure was analyzed using CD spectroscopy (**Figure 5**).

All four recombinant proteins run on SDS-PAGE at an apparent molecular weight of 43 kDa. This is consistent with previous published results (**Figure 4**) (Talkish et al. 2019). Additionally, all four protein preparations contain secondary structure as seen with CD spectra consistent with the presence of both alpha helix and beta sheet, as expected from the presence of Cus2's RRM fold which is comprised of a β-α-β-β-α-β fold (**Figure 5**).

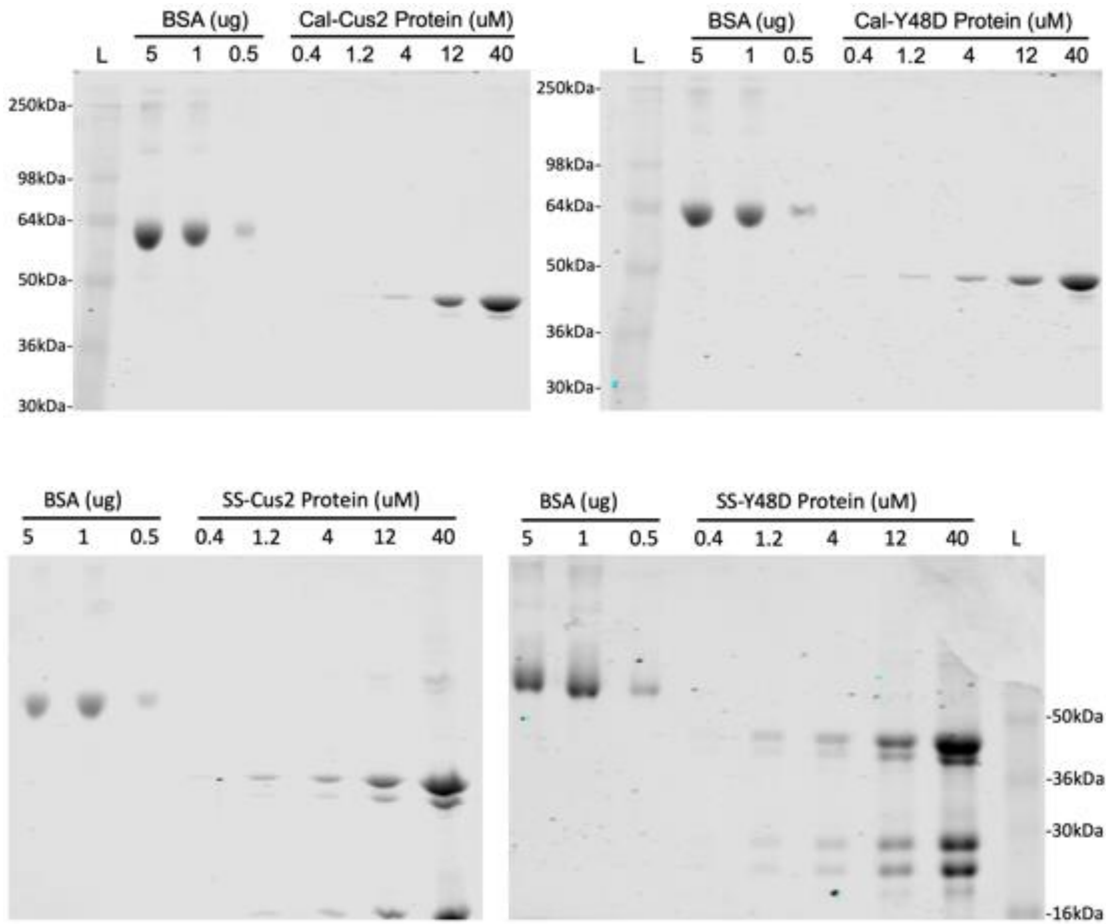

Figure 4: SDS-PAGE gels of purified proteins
Shown are protein gels of Cal-Cus2 and Cal-Y48D protein preps (top) or SS-Cus2 or SS-Y48D protein preps (bottom) at uM concentrations used for Kd measurements. Three lanes of BSA standards were run next to the Cus2/Y48D preps and are labelled by amount of micrograms loaded. Proteins were denatured by adding form dye, incubating at 95°C for 3 minutes, then loaded on a 1% SDS, 10% Bis:Acrylamide (37.5:1) gel and run in Tris-Glycine buffer (25mM Tris-Cl, 250mM Glycine). SeeBlue Pre-stained protein ladder was used and apparent molecular weights under Tris-Glycine buffer conditions are listed.

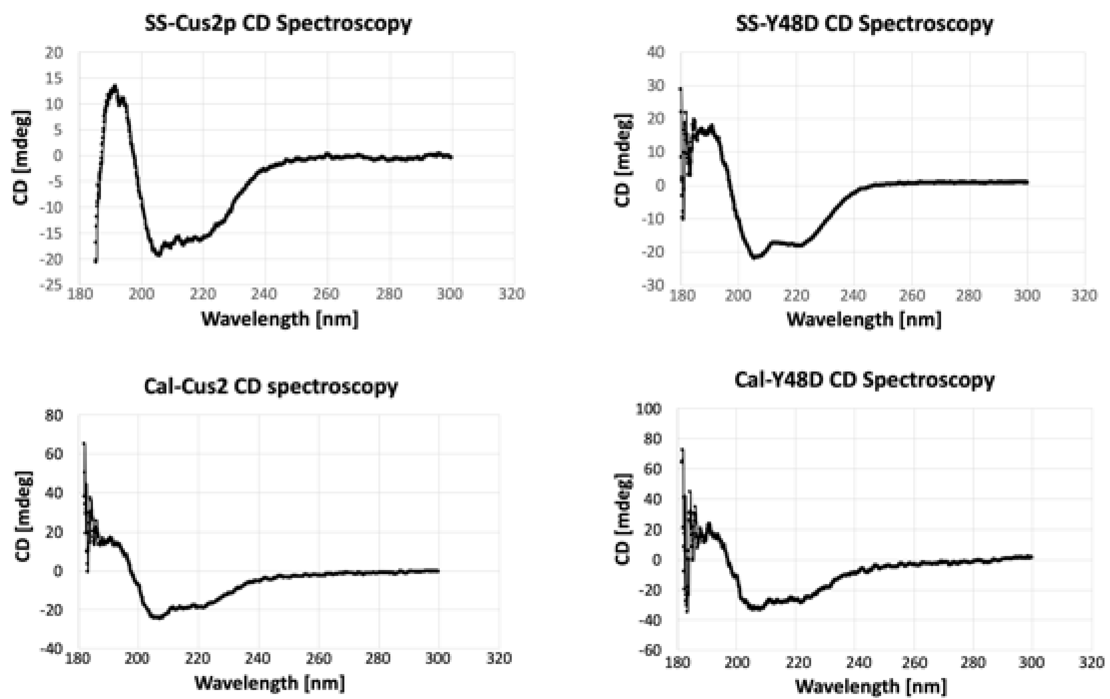

Figure 5: CD Spectroscopy Analysis of protein preps
Shown are spectra taken from SS-Cus2 (top left), SS-Y48D (top right), Cal-Cus2 (bottom left), and Cal-Y48D (bottom right) preps were diluted to 100ng/ul (3.1uM) in 1X EMSA Buffer and loaded on a 10um quartz cell. Signal was blanked with 1X EMSA Buffer prior to application of protein samples.

Do SS-Cus2 and SS-Y48D protein preps bind different conformations of U2 snRNA with different affinities?

The binding of Y48D to the IIc stable U2 snRNA forms a novel, and strong complex in an electrophoretic mobility shift assay (EMSA) (Rodgers et al. 2016). This evidence strongly aligns with the existence of a second site on Cus2, which binds U2 snRNA while the IIc conformation is stabilized. If such a binding site exists, the strength of the Y48D-IIc shift suggests that its binding affinity for IIc U2 might be stronger than the binding affinity of the canonical RRM. This hypothesis led me to ask the question; is binding between Y48D and IIc+ stronger than binding between the wild type protein and wild type U2? I therefore measured the dissociation constant (K_d) of wild type Cus2 (SS-Cus2) and Y48D (SS-Y48D) to the stem II containing region of wild type U2 snRNA (nucleotides G32-C124) as well as a U2 snRNA stabilized in its IIc form through mutations G53A, A95C & U97G (collectively called IIc+) (**Figure 6**). My results show that wild type Cus2 (SS-Cus2) binds wild type U2 with an apparent K_d of 4.5uM and to the IIc+ sequence with an apparent K_d of 4.8uM (**Figure 6**).

Binding of SS-Y48D protein preps to either RNA probe was tested next using the same native gel binding assay (Igel et al. 1998, Rodgers et al. 2016). Surprisingly, the SS-Y48D binds to the wild type U2 sequence with an apparent K_d of 18.5uM, and to the IIc+ U2 probe with a K_d of 15uM (**Figure 6**). As shown, the Y48D mutation alters the binding pattern to both RNA probes compare to Cus2's binding pattern.

Though the wild type protein reaches saturation near 10uM, complete binding of SS-Y48D to either RNA transcript was not seen even in the presence of 40uM protein.

The K_d measurement between SS-Cus2 and U2 compared to SS-Y48D and U2 indicates that binding between the wild type protein is stronger than binding of Y48D (4.5uM vs 18.5uM respectively). Additionally, binding of SS-Cus2 and SS-Y48D confers a different binding pattern in vitro, as seen by the different shifts in RNA (**Figure 6**). This data is consistent with a model in which Cus2 contains two binding sites for the RNA at separate locations. Although this data could result from weakening the existing RRM with the Y48D disruption, we would expect in such a case for the complex formed between each protein to be identical.

The SS-Cus2 binds to IIc+ RNA with a K_d of 4.8uM while the SS-Y48D protein binds the same RNA with a K_d of 15uM. The binding patterns seen in this assay are identical to those seen when incubating the proteins with a wild type U2 snRNA. This result implies that the binding of the Cus2 to its RNA target is based not on the conformation of RNA as previously shown, but rather on the presence (or lack of) a disrupted RRM.

Shown here, SS-Y48D can bind to both wild type U2 snRNA and IIc stable U2 snRNA. This interesting result contradicts previous studies of the Y48D mutant protein, in which the protein is unable to bind wild type U2 snRNA (Yan et al. 1998, Rodgers et al. 2016). One prominent difference between all previous studies of Y48D and this one is in the treatment of the protein-RNA sample. While all previous studies incubate protein and RNA together at 30°C for half an hour, samples in this study

were first incubated at 42°C for 5 minutes and then incubated at 30°C for another 25 minutes. It is possible that this heat shock has caused the Y48D protein to behave in a novel manner such that it can now interact with wild type U2 snRNA. It has been shown through FRET that wild type RNA interconverts between its two conformations, and it is possible that this increase in temperature allows for that interconversion to be in a higher dynamic state, so that the Y48D protein can bind the RNA in its particular IIc conformation. Alternatively, the increase in temperature might change the conformation of the protein such that the putative binding site in our model is now able to bind the wild type protein.

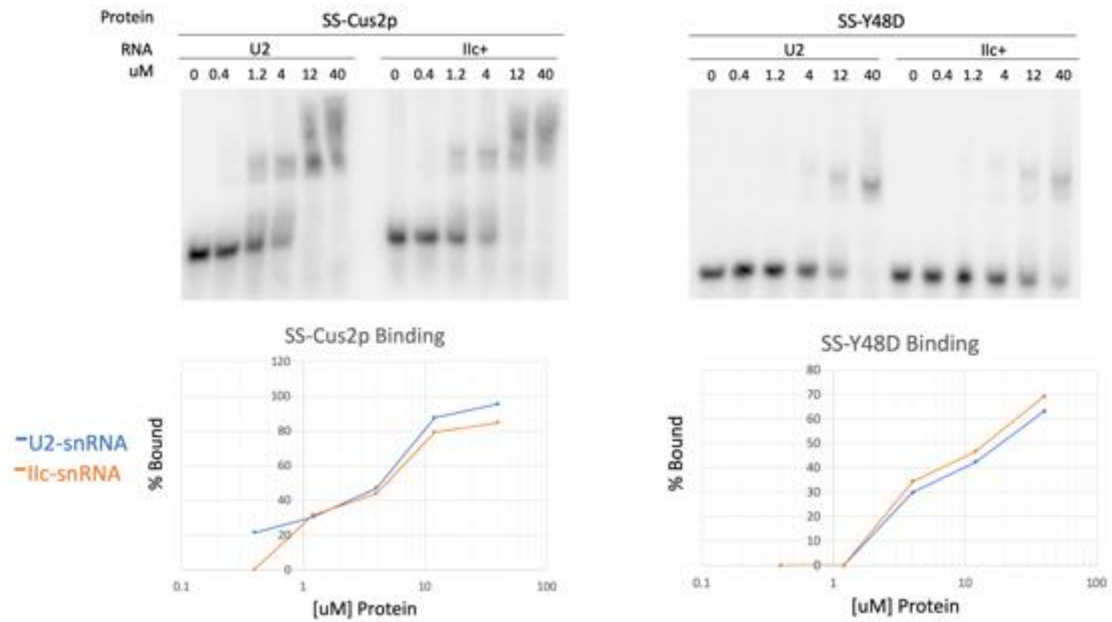

Figure 6: RNA Electrophoretic Mobility Shift Assays with SS- protein preps: Wild type U2 (U2) or stem IIc stabilized U2 (IIc+) RNA was incubated with increasing amounts of purified SS-Cus2 (left) or SS-Y48D (right) protein. Graphs indicate relationship between substrate bound versus protein concentration.

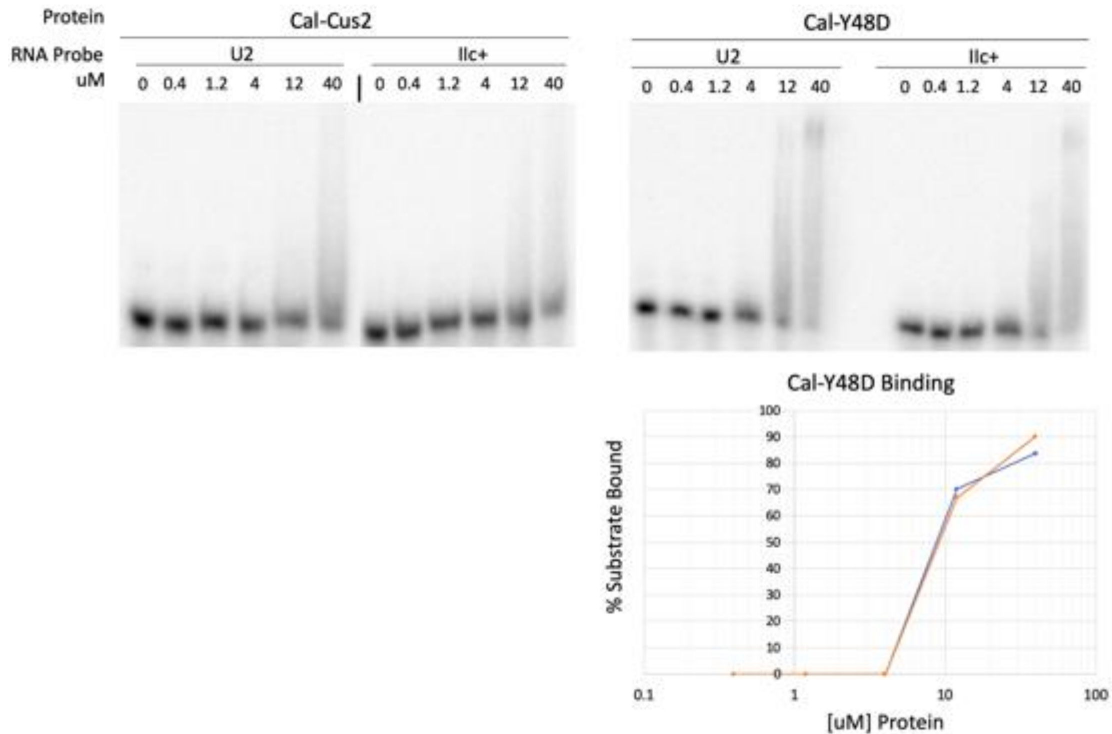

Figure 7: RNA Electrophoretic Mobility Shift Assays with Cal- protein preps

Wild type U2 (U2) or stem IIc stabilized U2 (IIc+) RNA was incubated with increasing amounts of purified Cal-Cus2 or Cal-Y48D protein. Graph indicates relationship between substrate bound versus protein concentration. Graphs indicating Cal-Cus2 binding have been omitted due to unreliable complex formation, and hence weak binding between this protein prep and either RNA probe.

Do both SS- and Cal- purified protein preps show identical U2 binding?

Next, I addressed the binding affinity of the Cal- proteins towards the wild type U2 or IIc stable U2 snRNAs to ensure consistency across different protein preps of Cus2. Proteins were once again diluted in 1X EMSA buffer to appropriate concentrations and confirmed through SDS-PAGE analysis. Electrophoretic mobility shift assays were carried out identically as before, utilizing the same transcripts as for SS-Cus2 and SS-Y48D.

Incubation of the same RNA in the presence of Cal-Cus2 and Cal-Y48D resulted in very little shift under the same binding conditions (**Figure 7**). The binding of Cal-Cus2 to wild type U2 snRNA could not be reliably determined since incubation with increasing concentrations of protein never resulted in the formation of a solid, well defined complex, but rather instead results in a scattered smear of RNA (**Figure 7**). The interaction between Cal-Y48D and wild type U2 snRNA was measured at a K_d of 8.8uM, while the K_d of Cal-Y48D to the IIc+ probe was determined to be a similar 9.0uM. A large complex can be seen when Cal-Y48D is in the presence of both RNA probes, but this complex is absent when the same RNA probes are allowed to interact with Cal-Cus2 (**Figure 7**). Contrary to previous results, this data indicates that the SS-Y48D and Cal-Y48D mutant proteins can both bind to wild type U2 snRNA. Additionally, it appears that each protein can bind to either confirmation of RNA with a nearly identical K_d values, indicating that the RRM disruption caused by the Y48D mutation is not enough to weaken RNA binding under these conditions.

Complexes formed with SS-Cus2 show native gel shifts that differ from that of SS-Y48D. However, each protein shows similar shifts between wild type and IIc stable U2, suggesting that the proteins differ in how they interact with RNA regardless of conformation. Though each protein does not show discrimination between U2 conformations, the proteins do show different binding activity depending on the presence of the Y48D disruption. The wild type SS-Cus2 protein binds wild type U2 nearly four times stronger than SS-Y48D, and it binds IIc stable U2 nearly three times stronger than SS-Y48D. This result is consistent with the Y48D disruption causing a change in the protein's binding ability, and it is possible that the increased binding of SS-Cus2 compared to SS-Y48D is a result of the wild type protein containing multiple binding sites.

CHAPTER 3

EFFECTS OF DISRUPTING CUS2'S RRM ON YEAST GROWTH

In *S. cerevisiae*, colony growth is commonly used as an indicator of overall splicing activity in the cell. The disruption of stem IIa of U2 with the G53A mutation causes an inhibition of pre-spliceosome formation, and therefore overall splicing, leading to a decrease in cell growth (Yan et al. 1998). Under cold conditions, this becomes nearly lethal. However, CUS2 is able to rescue this cold sensitivity by binding to U2 and refolding it from its stem IIc to its stem IIa form. Previous studies show that the interaction between the Y48D mutant of Cus2 can only bind IIc stable RNA to form a novel shift in binding assays. The apparent strength of the IIc stable U2 and Y48D protein interaction suggests that the protein may bind U2 and constrain the RNA from refolding, preventing it from associating into the pre-spliceosome. If this is true, the presence of Y48D in the presence of only a IIc stable U2 should inhibit splicing and perturbed cell growth. Furthermore, this growth deficiency should be prominent in the cold where U2 rearrangement is inefficient without splicing factors. I therefore expect to see a dominant negative phenotype in cells containing the Y48D mutant and an endogenous IIc+ U2 over cells with no Cus2 or Y48D at all.

<u>Will the Y48D mutation have a dominant negative phenotype in the presence of only IIc stable U2 snRNA?</u>

To test this, I made strains SS01 by transforming *S. cerevisiae* strain BY4741 CUS2Δ, TRPΔ with a linear URA3-Gal-U2 *Hind*III fragment to recombine with and replace endogenous U2 with a glucose repressible U2 gene. Repression of endogenous U2 allows for examination of growth when U2, or any variant, is

expressed on a plasmid. SS01 was then transformed with pRS315 vector containing either wild type U2, G53A-U2 or WCUP-U2. Both WCUP and G53A mutations of U2 stabilize the IIc stem loop; WCUP acts to increase stem IIc base pairing interactions, while G53A acts to disrupt the IIa stem loop, thereby shifting equilibrium of the RNA to its IIc conformation. Retention of these plasmids was achieved through leucine expression on pRS315-U2, pRS315-WCUP or pRS315-G53A vector and selecting on plates containing SCgal -Leu media. Each strain was then transformed with vector pRS314-CUS2, pRS314-Y48D, or a "blank" pRS314 plasmid containing neither protein gene, and selected for on plates containing SCgal -Leu -Trp media. Double transformants were grown on plates with SCgal -Ura -Trp -Leu, then re-streaked on SCgal -Ura, -Trp, -Leu for single colony formation, and colonies were then used to inoculate 5mL overnight liquid cultures in YPgal liquid media. Cultures were diluted down to an equal OD600 value of 0.100 in YPgal. Ten-fold serial dilutions were then spotted on SCD -Ura -Trp -Leu, and grown at indicated temperatures (**Figure 10**).

Cells expressing Y48D protein instead of wild type Cus2 did not show a significant growth defect in either a wild type U2 background or a WCUP-U2 background at 37°C, 30°C, 26°C, and 18°C. Cells containing a WCUP-U2 and not containing either protein show similar growth to cells containing wild type U2. Interestingly, cells containing WCUP-U2 but lacking any Cus2 are able to grow at 18°C, an effect which has not been observed prior to this study. Next, I tested the impact of the Y48D protein in cells expressing G53A-U2.

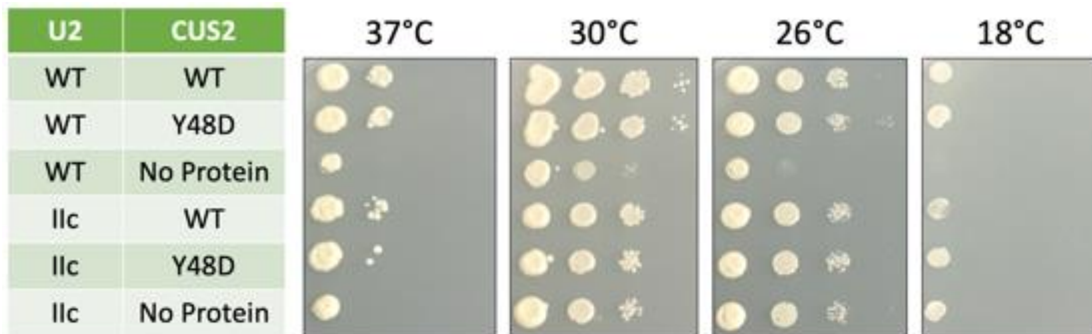

Figure 8: Genetic growth assay of U2 mutant WCUP and Cus2 or Y48D

Presence of Y48D in presence of IIc stabilized using the WCUP mutations (A95C & U97G) causes inhibited growth in comparison to a wild type U2 background only at 37°C. Growth of ten-fold dilutions of strain SS01 containing wild type or IIc stable U2 are seen in presence of Cus2 protein, Y48D protein, or a no protein control at various temperatures shown (37°C, 30C, 26°C, and 18°C).

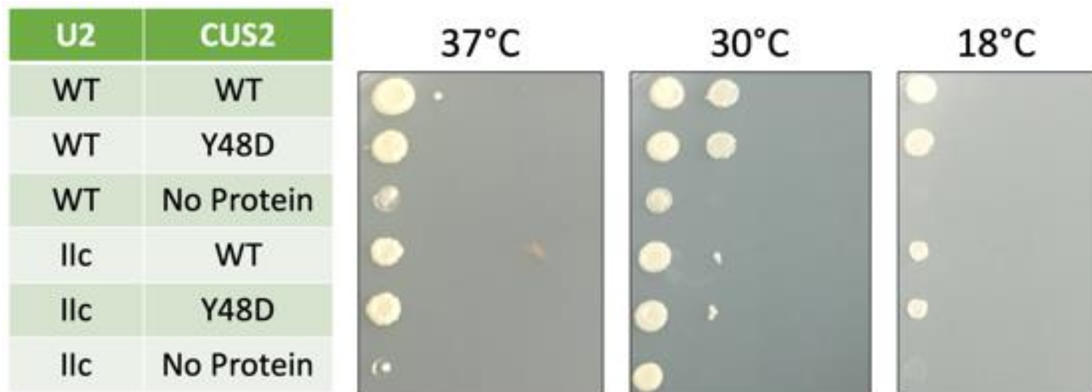

Figure 9: Genetic growth assay of U2 mutant G53A and Cus2 or Y48D

Presence of Y48D in presence of IIc stabilized with the G53A mutation does not cause inhibited growth in comparison to a wild type U2 background. Growth of ten-fold dilutions of strain SS01 containing wild type or IIc stable U2 are seen in presence of Cus2, Y48D protein, or a null, no protein control at various temperatures shown (37°C, 30C, and 18°C).

Strain SS01 expressing wild type U2 along with Cus2 protein grows at a rate similar to cells expressing the Y48D protein. A similar growth pattern is seen in strains containing G53A-U2: Cus2 and Y48D expression have identical growth patterns at all temperatures tested. However, incubation of yeast with the G53A mutation and no protein at 18°C is cold sensitive. This decrease in growth is absent at 30°C where U2 containing the G53A mutation can interconvert between its two conformations thermodynamically. Notably, the complete absence of protein in wild type U2 strains causes an observable decrease in growth at all temperatures. This can be interpreted as a dependence on Cus2 that was not previously observed. This proposes a model whereby repression of chromosomal U2, and replacing it with a stem IIc stable U2 on a plasmid, requires the CUS2 gene for proper splicing to occur.

One prediction of our model is that the Y48D mutant might be capable of causing a dominant negative phenotype on growth in cells which express only a IIc stable U2 snRNA. Growth in cells transformed with Y48D and expressing IIc stabilized through the WCUP mutation or the G53A mutation is not significantly inhibited, indicating that Y48D does not cause a dominant negative phenotype.

Since Cus2 activity is less necessary under higher temperatures, strains SS01-U2, SS01-WCUP, and SS01-G53A containing the blank pRS314 plasmid were expected to grow at 26°C, 30°C, and 37°C, but were expected to grow at a slower rate at 18°C. While this is seen in the G53A strains, the WCUP strain is seen growing as well as strains containing Cus2 and Y48D at 18°C. This result is inconsistent with the WCUP mutation causing stem IIc to be hyper stabilized, and causing cold sensitivity

(Perriman & Ares 2007). Due to this strain behaving in a manner consistent with wild type U2 presence, and the wild type U2 strain lacking protein behaving in a manner consistent with a WCUP induced growth defect, the comparison between these controls and the Cus2 and Y48D containing strains is inconclusive.

CHAPTER 4

SUMMARY OF RESULTS

Biochemical EMSA Results

Here I have tested a possible model for splicing factors CUS2's activity during splicing and complied experiments that test binding interactions between U2 RNA and the wild type protein or its binding mutant Y48D.

The difference in complexes seen when SS-Cus2 and SS-Y48D interact with the same U2 snRNA indicates that separate binding sites are responsible for the in vitro interaction. Thus, it is possible that U2 snRNA refolding in cold temperatures depends on the presence of Cus2 protein containing both its RRM and the putative binding site. In the cell, and under cold or otherwise unfavorable growth conditions for yeast, it is possible that the binding between Cus2 to U2 requires both binding sites present to ensure capture of the correct snRNA to prioritize refolding of critical splicing components.

The binding between the SS-Y48D protein and the wild type U2 snRNA suggests that Cus2 containing a disrupted RRM may bind to U2 snRNA so long as the dynamics in the RNA are increased. This is consistent with Cus2 protein being responsible for identifying misfolded, or mutated U2 snRNA in the cell. In the wild, it is possible that weaker interactions in its stem II region which have not been specifically tested require the binding of Cus2 to refold mutated U2 snRNA correctly. Since this study addresses only two different U2 snRNA variants, it is still unknown whether Cus2 becomes an essential gene, even at ideal temperature, when U2 snRNA contains extremely weak secondary structures.

Genetic effects of Cus2 and Y48D in the presence of U2 stem II mutants

My results indicate that Y48D in the presence of only a stem IIc stable U2 snRNA does not cause a dominant negative phenotype. This result, along with the K_d measured between the Y48D protein and U2 in both its conformations, make the case that binding between the proposed second RNA binding site and U2 is less efficient than previously thought if in fact it even exists. However, since Cus2's function becomes more critical in colder conditions, the strength of this recognition may be aided by the thermodynamics inside the cell. EMSA analyses of the protein at temperatures below 30°C may yield results indicating higher binding between the Y48D protein in comparison to the wild type Cus2 protein.

A possible explanation to the unexpected growth activity seen in the WCUP containing strains and wild type U2 containing strains in the absence of proteins may be related to the background of the "SS01" strain in contrast to the "RP01" strains used in previous studies. In 1998, the strains designed to test Cus2 deletions in fact used a disruption of the CUS2 gene by insertion of the HIS3 gene. In such a disruption allele, it is theoretically possible for residual expression of Cus2 protein fragments. It is therefore possible that the growth defects seen at 18°C using this previous disruption is attributed to the presence of Cus2 protein fragments in the presence of a IIc-stabilized RNA.

The possibility of a Cus2 fragment being expressed in previous studies leaves questions unanswered regarding Cus2 protein expression. It also proposes the possibility that past cold sensitivity is seen as a result of such a Cus2 fragment. To

test this, a strain expressing this C-terminal peptide in the presence of the WCUP U2 mutation can be incubated at varying temperatures as conducted here.

This interpretation raises the question of whether such a C-terminus portion of the Cus2 protein can bind to IIc stable U2 and/or wild type U2 snRNA. Further classification of the role of the C-terminus of CUS2 and the role of the two suppression enhancing mutations D282N, and L284F, is needed. The results presented here suggest that previous analyses of Cus2 may have observed results attributed to the C-terminus of the protein, though this needs to be confirmed. It is possible this region of CUS2 plays a more critical role not explored in previous CUS2 studies. EMSA analysis using this same C-terminus portion of the protein and various U2 snRNA conformations would provide evidence of such an interaction.

MATERIALS AND METHODS

Deoxyoligonucleotides

PCR Oligonucleotides:

(1) T7-U2bp-A52:
5’-AAAAGGGCCCTAATACGACTCACTATAGGGTGTAGTATCTGTTCTTTTC
A-3’

(2) U2-reverse-Sm:
5’- GGTGCCAAAAAATGTGT-3’

(3) T7-U2-G53A:
5’-TAATACGACTCACTATAGGGTGTAGTATCTGTTCTTTTCAATGTAACAA
CTGAAATGACCTCAATGAGGCTCATT-3’

(4) U2-Sm-Rev-WCUP:
5’- GGTGCCAAAAAATGTGTATTGTAACAACTGAAAAGGTAATGAGCCTC
ATTGAGGTCA-3’

RNA substrates:

(5) Wild type U2 transcription template:
5’-AAAAGGGCCCTAATACGACTCACTATAGGGTGTAGTATCTGTTCTTTTC
AGTGTAACAACTGAAATGACCTCAATGAGGCTCATTACCTTTTAATTTGTT
ACAATACACATTTTTTGGCACC-3’

(6) IIc+ U2 PCR transcription template:
5’-AAAAGGGCCCTAATACGACTCACTATAGGGTGTAGTATCTGTTCTTTTC
AATGTAACAACTGAAATGACCTCAATGAGGCTCATTACCTTTTCAGTTGTT
ACAATACACATTTTTTGGCACC-3’

*Notable differences between WT and IIc+ sequences (G53A, A95C and T97G) are underlined.

Yeast Strains and Plasmids

Yeast transformations were carried out as previously described (Hill 1991). The CUS2 gene was deleted from strain BY4741; MATa, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, trp1Δ0 by transformation with a PCR fragment encoding the NAT gene with ends homologous to the genome just outside the CUS2 open reading frame. Strain SS01 was made by transforming BY4741 Trp1Δ, CUS2Δ with a linear *Hin*dIII fragment of U2-URA3 GAL-U2 gene and then selecting on galactose medium lacking uracil. Growth of yeast was performed using standard procedure (Sherman 1991). Colonies were then re-streaked on SCD -Ura, as well as on 5FOA to confirm chromosomal U2 knock out and presence of the U2-URA-GAL insert, respectively (data not shown). SS01 was transformed with Leu2 selective plasmids containing the U2 wild type gene, U2 G53A, or U2 WCUP and grown on SCgal -Leu. Then these strains were subsequently transformed with pRS314-Cus2, pRS314-Y48D, or pRS314 plasmid and selected for on SCgal -Leu -Trp -Ura media. Double transformants were re-streaked on SCgal -Leu -Trp -Ura and then colonies were streaked onto SCD -Leu -Trp -Ura to repress chromosomal U2 expression.

***In vitro* transcription**

Transcripts (5) and (6) utilized in binding assays were obtained by PCR-amplifying the T7 promoter onto T33 of LSR1 using oligos (1, 2) and (3, 4) for wild type U2 or IIc+ U2 snRNA respectively from the pUC13 plasmid (pUC13-U2). RNA probes were radiolabeled by transcribing with T7 RNA polymerase from the MEGAscript Kit (Ambion [Life Technologies]) and ^{32}P-gamma-UTP at 37°C for 4 hours. dNTP concentrations were adjusted to account for introduction of radiolabeled UTP. Transcripts were purified on a 6% acrylamide (19:1 Bis: Acrylamide) gel for 40 minutes at 20W, then eluted into RNA elution buffer overnight and phenol:CHCL3 extracted and ethanol precipitated the following day. All transcripts were resuspended in dH2O, then measured on a scintillation counter to determine concentration.

Protein expression and purification

Rosetta (DE3) *E.coli* cells transformed with pET24- Cus2-His were grown overnight in 4mL LB/Km (25ug/ml), then overnight culture was used to inoculate 1L of LB/Km until an OD 0.600 was reached. Cells were then induced with 1mM IPTG for 4-5 hours at 30°C. After induction, cells were harvested by centrifugation at 5500 RCF for 35 minutes at 4°C, and pellets were stored at -80°C. Cells were later resuspended in 35ml lysis buffer (500mM NaCl, 50mM HEPES pH 7.4, 3mM BME), supplemented with Lysozyme (1mg/mL), PMSF (100uM final), and then sonicated for eight, 30 seconds pulses at 4°C and 60kHz. Lysate was then diluted 1:1 with lysis buffer and loaded onto a 1mL or 5mL Nickel-ion column HiTrap Chelating HP column (GE Healthcare Life Sciences) in 5% Imidazole and eluted with a gradient

from 4-80% elution buffer (lysis buffer containing 500uM Imidazole) over 15 column volumes.

Then proteins were eluted further with a gradient from 80-100% elution buffer over 5 column volumes. Elution fractions were run on 15% SDS-PAGE and fractions containing Cus2-His were pooled, and then dialyzed against 200mM NaCl, 25mM HEPES pH 7.4, 3% Glycerol, with added 0.5mM DTT, 0.1mM PMSF, and 0.2mM EDTA. SS-Cus2 and SS-Y48D protein preps were not purified any further, while Cal-Cus2 and Cal-Y48D preps were equilibrated, loaded and washed onto a Subtractive Q-HiTrap column with 200mM NaCl, then eluted from 10-70% elution buffer (1M NaCl, 25mM HEPES pH 7.4 + 0.5mM DTT) over 15 column volumes, then 70-100% elution buffer for 5 column volumes, and cleaved with 100% elution buffer for 3 more column volumes. 0.1mM PMSF was added to fractions containing protein, and they were then concentrated using a 10kDa MWCO to ~1mL and stored on ice until gel filtration or stored at -80°C for storage. Finally, the Superdex-75 column was washed with 1 column volume of dH2O and equilibrated with wash buffer (200mM NaCl, 15mM HEPES pH 7.4, 0.2mM DTT), and then concentrated samples were injected and washed with 5mL wash buffer. Wash buffer was run for 1 column volume, then peak fractions were run on 12.5% SDS-PAGE. Fractions with concentrations of ~100uM or more were frozen in 100uL aliquots and not pooled. Samples were concentrated using 10kDa MWCO to 1mL and frozen in 100uL aliquots. Purification of SS-Cus2 was carried out using the AKTA Prime, and SS-Y48D was purified on the AKTA Pure. Cal-Cus2 and Cal-Y48D proteins were purified at the QCB MacroLab facility at UC Berkeley.

Electrophoretic Mobility shift assay

EMSA was carried out essentially as previously described (Igel et al. 1998). 20fmol of ^{32}P-radiolabeled RNA probe was incubated with increasing amounts of SS-Cus2, Cal-Cus2, SS-Cus2, or SS-Y48D (mutant) protein in 1X EMSA Buffer (125mM KCl, 20mM HEPES pH 7.9, 1mM EDTA, 5% Glycerol, 0.1% Triton X-100, 1mM DTT) with 0.3U RNasin Ribonuclease inhibitor (Pomega) in 10uL reactions which were incubated at 42°C for 5 minutes, then 30°C for 25 minutes. 2uL of loading buffer (50% Glycerol, 0.1% Xylene Cyanol) was added to reactions, then loaded on a native 5% Acrylamide (60:1), 10% Glycerol gel and run for 4hr at 4°C. EMSAs were visualized by exposure to a phosphorimaging screen for 16 hours, and data was analyzed using Image Quant software (GE Healthcare Life Sciences).

Protein analysis

Bradford assays were used to measure concentrations of each protein prep as described in the Bio-Rad Protein Assay (Bradford 1976). Protein standards were made by adding 200ul of Bradford reagent to 800ul of 2, 3, 5, 9, and 10ug/ml BSA, then incubated at room temperature for 5minutes, and absorbance was measured at

595nm. This was used to plot a standard curve to calculate concentrations from absorbance of each protein prep.

All protein preparations were run on a 1% SDS, 10% acrylamide (37.5: 1, Bis: Acrylamide) protein gel in Tris-Glycine running buffer (25mM Tris-Cl, 250mM Glycine) along with BSA standards (Sigma-Aldrich) to reassess accuracy of concentrations and to assess purity. Proteins were visualized by staining with Coomassie blue for 1 hour at room temperature or overnight at 4°C, and then visualized using Image Studio Lite software (LI-COR Biosciences).

To assess the presence of intact secondary structure each prep was also analyzed using CD Spectroscopy. SS-Cus2, SS-Y48D, Cal-Cus2, and Cal-Y48D preps were diluted to 100ng/ul in EMSA buffer (125mM KCl, 20mM HEPES pH 7.9, 1mM EDTA, 5% Glycerol, 0.1% Triton X-100, 1mM DTT), then loaded onto a 10um quartz cell for visualization. The same 1X EMSA Buffer was used as a blank to account for expected background signal from Triton X-100 and DTT.

Bioinformatics

Alignment of CUS2 gene to orthologs was done using the EMBL-EBI Clustal Omega (v.1.2.4) multiple sequence aligner at https://www.ebi.ac.uk/Tools/msa/clustalo/ accessed on April 20, 2019 (Maderia et al. 2019). Input sequences were in FASTA formatting and can be obtained from NCBI using Sequence IDs (aligned to genomes) as follows: NP_014113.1 (*S. cerevisiae*), NP_596826.1 (*S. pombe*), NP_001156752.1 (*H. sapiens*), NP_490765.1 (*C. elegans*), and NP_649313.1 (*D. melanogaster*), all with default algorithm parameters. These organisms were chosen as representatives of both a wide evolutionary range in Eukaryotes, as well as a sampling of highly studied model organisms. Output selected was “ClustalW with character counts”, and setting used were as follows: Dealign Input Sequences: no, MBED-Like Clustering Guide-Tree: yes, MBED-Like Clustering Iteration: yes, Order: aligned. All other settings were set to default.

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