

1-2009

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Maeder, C., Kutach, A. K., & Guthrie, C. (2009). ATP-dependent unwinding of U4/U6 snRNAs by the Brr2 helicase requires the C terminus of Prp8. *Nature Structural and Molecular Biology*, 16(1), 42-48. doi:10.1038/nsmb.1535

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Author manuscript

Nat Struct Mol Biol. Author manuscript; available in PMC 2009 July 08.

Published in final edited form as:

Nat Struct Mol Biol. 2009 January ; 16(1): 42–48. doi:10.1038/nsmb.1535.

ATP-dependent unwinding of U4/U6 snRNAs by the Brr2 helicase requires the C-terminus of Prp8

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Summary

The spliceosome is a highly dynamic machine requiring multiple RNA-dependent ATPases of the DExD/H-box family. A fundamental unanswered question is how their activities are regulated. Brr2 function is necessary for unwinding the U4/U6 duplex, a step essential for catalytic activation of the spliceosome. Here we show that Brr2-dependent dissociation of U4/U6 snRNAs *in vitro* is activated by a fragment from the C-terminus of the U5 snRNP protein Prp8. In contrast to its helicase-stimulating activity, this fragment inhibits Brr2 U4/U6-dependent ATPase activity. Notably, U4/U6 unwinding activity is not stimulated by fragments carrying alleles of *prp8* that in humans confers an autosomal dominant form of retinitis pigmentosa. Because Brr2 activity must be restricted to prevent premature catalytic activation, our results have important implications for fidelity maintenance in the spliceosome.

Keywords

pre-mRNA splicing; spliceosome; retinitis pigmentosa; protein co-factor

Introduction

The spliceosome is a highly dynamic RNA-protein machine requiring the ordered binding and release of 5 small nuclear ribonucleoprotein particles (snRNPs) to the intron-containing pre-mRNA1. Following recognition of the 5' splice site by base-pairing to U1 snRNA and the intron branchpoint sequence by base-pairing to U2 snRNA, the U4/U6·U5 triple snRNP complex joins the assembling spliceosome. In this complex, U4 snRNA is extensively base-paired to U6 snRNA. Despite the presence of all 5 essential snRNAs, this form of the spliceosome is catalytically inactive; the two sequential trans-esterification reactions require the unwinding of U4 from U6 and the coordinated release of U1 snRNP. This strategy presumably ensures that catalytic activation is restricted until the appropriate intronic signals have been accurately identified. After the completion of the chemical steps, the spliceosome disassembles and the snRNPs must be recycled for the next round.

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Aside from the binding of the U1 snRNP to the 5' splice site, each step in this dynamic cycle requires ATP and one or more DExD/H RNA-dependent ATPases, a large protein family whose members share a ~300 amino acid domain with eight highly conserved sequence motifs^{2,3}. The founding member of this family is eIF4A, a DEAD-box protein that is thought to use the energy of ATP hydrolysis to unwind base-paired RNAs⁴. In other cases, the ATPases are thought to promote the displacement of proteins from a bound RNA^{2,3}. In budding yeast, eight DExD/H-box proteins are required for splicing: Sub2, Prp5, Brr2, Prp2, Prp28, Prp16, Prp22 and Prp43 (ref. 1). Several of these RNA-dependent ATPases have been shown to possess weak in vitro RNA unwinding activity⁵⁻¹¹; in the case of Prp43, this activity has been shown to be stimulated by a protein co-factor, Ntr1 (ref. 10). Further progress has been hindered by the absence of precise information about the targeted rearrangements. Since several of these DExD/H-box proteins have been shown to play an important role in fidelity maintenance¹²⁻¹⁶, an understanding of how their activities are timed is of crucial importance.

Many of the DExD/H-box proteins have been shown to be only transiently associated with the spliceosome and their regulation might thus be accounted for, at least in part, by the timing of their associations¹. In contrast, Brr2 is an integral component of the U5 snRNP, where it is tightly complexed with Prp8 and Snu14. Brr2 is an atypical family member in that it contains two helicase-like domains^{17,18}. The C-terminal domain differs substantially from consensus motifs and several mutations failed to confer a biological phenotype¹⁹. The N-terminal domain contains a canonical DEIH-box motif and a mutation in the ATP binding region of the budding yeast protein confers a cold-sensitive growth phenotype²⁰. Notably, the *brr2-1* allele, a mutation in the N-terminal DEIH-box motif, eliminates the ATP-dependent unwinding of U4 from U6 in vitro²¹. The human Brr2 protein has been shown to unwind U4/U6 duplexes in vitro, but this reaction required a large excess of protein to RNA and long incubation times⁶.

Unwinding of U4 from U6 is essential for the catalytic activation of the spliceosome to allow the re-folding of U6 with U2 snRNA²². Moreover, it has recently been shown that Brr2 must function again at the end of the cycle, in this case to promote dissociation of U2 from U6 (ref. 23). Thus the regulation of Brr2 activity must be central to the fidelity of splicing, to ensure that neither catalytic activation nor spliceosome disassembly is premature. Both of these Brr2-dependent steps also require the function of Snu14, an EF2-like GTPase^{23,24}. Extensive genetic and biochemical evidence has also implicated Prp8 in the function of Brr2 and Snu14 (reviewed in refs. 25,26). In particular, both the ATPase and the GTPase interact with the N and C termini of Prp8 (refs. 27-29), itself the largest protein in the spliceosome. Interestingly, the C-terminal region of Prp8 contains a Jab1/MPN domain²⁶ which interacts with ubiquitin in vitro³⁰ although it lacks the catalytic residue required for deubiquitinase function. As shown in recent crystal structures of the ~300 C-terminal amino acids of human and yeast Prp8, the N- and C-terminal extensions of the Jab1/MPN motifs form alpha helices that wrap around the compact core^{28,29}. Remarkably, the very C-terminus forms an unstructured tail that contains the sites of 16 independent mutations that lead to the onset of retinitis pigmentosa (RP), a degenerative eye

disease^{31,32}. The introduction of several RP alleles into budding yeast Prp8 weakened 2-hybrid and co-immunoprecipitation interactions with Brr2 (ref. 32).

Here we show that a ~600 amino acid region from the C-terminus of Prp8 interacted with full-length Brr2 and efficiently stimulated its ATP-dependent unwinding of a synthetic U4/U6 duplex in *Saccharomyces cerevisiae*. This stimulation was lost or substantially weakened when the Prp8 fragment contains an RP mutation, even when interaction of Brr2 with Prp8 was not substantially reduced. Interestingly, in contrast to its stimulation of Brr2 helicase activity, the wild-type Prp8 fragment actually inhibited the RNA-dependent ATPase activity of Brr2. These results suggest that Prp8 regulates the timing of unwinding activity of Brr2.

Results

Prp8-CTF interacts with Brr2 in vitro

While little is known about the domain structure of Prp8, genetic and yeast two-hybrid studies have shown that both the N- and C-termini of Prp8 interact with Brr2 (ref. 33). To determine whether the interaction between the C-terminal region of Prp8 and Brr2 is direct, we performed binding assays between Prp8 and Brr2. To isolate fragments of the insoluble 2413 amino acid Prp8 for biochemical studies, we performed protease hypersensitivity mapping to determine discrete domain boundaries (Supplementary Fig. 1). Using our defined fragments, we were able to express and purify from *E. coli* a soluble, recombinant 607 amino acid fragment of Prp8 (1806-2413 aa) N-terminally tagged with glutathione-S-transferase (GST) (Prp8-CTF). Prp8-CTF encompasses the RNase H and Jab1/MPN domains (Fig. 1A). Recent crystal structures for the N-terminal half of our fragment determined a β -finger domain surrounded by an RNase H domain, which lacks its active site residues³⁴⁻³⁶ and the C-terminal half of our fragment confirmed the presence of a Jab1/MPN-like fold^{28,29}.

In order to examine the interaction between Prp8-CTF and Brr2, we performed GST-pull down experiments of Prp8-CTF and full-length Brr2, which was purified from *S. cerevisiae* overexpressing TAP-tagged Brr2 (ref. 37). We purified Brr2 under high salt conditions (750 mM NaCl) in order to disrupt interactions with spliceosomal proteins. Western blotting of 1 μ g of purified Brr2 failed to detect either of the U5 snRNP proteins Prp8 or Snu14. Brr2 was able to specifically associate with Prp8-CTF and not GST alone (Fig. 1B) in this purified system under lower salt conditions, demonstrating a direct interaction between these proteins, as previously observed by yeast two-hybrids, co-immunoprecipitation, and genetics³³. Additionally, Brr2 harboring the *brr2-1* helicase domain mutation²⁰ was able to associate with Prp8-CTF to the same extent, indicating that the interactions between these proteins is not affected by this mutation (Fig. 1B).

RP mutations effects growth and interactions with Brr2

Several mutations that are linked to the human degenerative eye disease RP are located in Prp8-CTF in a region C-terminal to the Jab1/MPN domain (Fig. 1A). In vitro splicing defects were found for several of the mutations³² suggesting that they are disruptive to the

splicing machinery. To determine the effects of these RP mutations on viability, we introduced plasmid-borne wild-type and RP-mutant versions of full-length *PRP8* to a strain in which the chromosomal copy of *PRP8* has been deleted. We observed a range of growth phenotypes for the series of alleles (Fig. 2A). As seen previously, strains containing 2 of the 5 alleles (H2387P and H2387R) are dead or severely sick at higher temperatures³².

To compare the effects on growth with the effects on Prp8's interactions with Brr2, we introduced the series of five RP mutations into our Prp8-CTF constructs. We were able to express and purify soluble recombinant Prp8-CTF mutant proteins from *E. coli*. However, the yield of the H2387P and H2387R mutant fragments was approximately ten times lower than the wild-type fragment suggesting that these fragments are less soluble or stable than the wild-type fragment. We then examined their ability to directly interact with Brr2 using our GST-pulldown interaction assay (Fig. 2B). Equimolar amounts of proteins are used in all interaction assays. In order to illustrate the hierarchy of Brr2 binding seen among the Prp8 RP mutants, we are showing the wild-type signal for Prp8 at saturation.

Although the strains with F2382L, R2388G or R2388K mutant versions of *PRP8* grew similarly to the wild type, we see weakened interactions of the corresponding mutant Prp8-CTFs with Brr2. However, H2387P or H2387R mutations in *PRP8* strains behaved quite differently. The *prp8-H2387P* mutant displayed slightly reduced growth at 30°C, and extreme sickness at 37°C. The *prp8-H2387R* mutant was extremely sick at 30°C, and inviable at 37°C. For both of these mutations, we observe only background level of Brr2 binding. These findings indicate the RP region of yeast Prp8 is important for Prp8's function. The most severe growth phenotype corresponds with the most severe Prp8-CTF-Brr2 interaction defect, but this does not rule out that the RP mutations result in phenotypes due to loss of some other function (see below).

The fact that mutations resulting in no interaction between Brr2 and Prp8-CTF in vitro are viable at all suggests one of two possibilities. First, the in vivo interaction is not essential for cell viability. Second, although our in vitro interaction assay does not detect binding of Brr2 to Prp8-CTF with either H2387 mutation, a more likely possibility is that the mutants retain some affinity for Brr2 in vivo. In support of the latter scenario, we have observed that addition of a C-terminal epitope tag to PRP8 exacerbates some of the RP-mutant phenotypes observed in yeast, although it has no effect on growth in a wild-type strain (data not shown and ref. 32). With an epitope tag, the H2387 mutants are inviable, and both of the R2388 mutants display temperature sensitivity. This is notable in light of the finding that one of the characterized RP mutations in humans results in conversion of stop codon to leucine, resulting in a 41 amino acid extension of the Prp8 polypeptide³⁸, further suggesting that inserting an epitope tag at the C-terminus of Prp8 is akin to introducing an RP mutation.

Prp8-CTF stimulates Brr2's helicase activity

Genetic interaction studies have suggested that Prp8 negatively regulates Brr2-dependent RNA unwinding events during the splicing cycle^{39,40}. Having established a direct interaction between Prp8-CTF and Brr2, we wanted to investigate what effect this interaction may have on Brr2's intrinsic dsRNA helicase and RNA-dependent ATP hydrolysis activities. Because other studies suggest that U4/U6 snRNA is a native substrate

of Brr2 (refs. 6, 21), we also chose to use U4/U6 snRNAs as a substrate in our unwinding assays.

U4 and U6 snRNAs were transcribed from a T7 promoter, purified and freshly annealed in the presence of Mg^{++} prior to each experiment. In addition to a full-length U6, we used a synthetic U6 snRNA (U6syn) containing nucleotides 22-112 (Fig. 3A), because the first 21 nucleotides of U6 have been shown to be dispensable for splicing *in vitro*⁴¹. After observing that full-length U6 and our U6syn behaved identically in our experiments (data not shown), we used U6syn for our remaining assays. Brr2 (25nM) and Prp8-CTF (250 nM) were combined with hybridized snRNAs (200nM) and pre-incubated at 30°C (see Materials and Methods). Helicase reactions were initiated by addition of ATP and then incubated at 30°C for the course of the reaction. At the time indicated, samples from each reaction condition were withdrawn and helicase activity halted by the addition of excess EDTA to chelate Mg^{++} ions (Fig. 3B). Unwound RNAs were separated by native gel electrophoresis and U4 snRNA was detected by a fluorescently labeled anti-U4 snRNA oligonucleotide⁴².

We first examined the ability of purified Brr2 alone to unwind U4/U6 snRNAs (Fig. 3b,c) in our *in vitro* system. Over the course of one hour we observed less than 20% of U4/U6 unwound to U4 and U6 (Fig. 3B). This is consistent with Lagerbauer et al. who showed that the reaction is not particularly efficient since a 100:1 ratio of enzyme:substrate incubated for one hour at 40°C does not completely unwind U4/U6 snRNA or a second dsRNA unrelated to splicing⁶. Strikingly, Brr2 in the presence of ten fold molar excess Prp8 was able to fully unwind the U4/U6 snRNA duplex within 30 minutes at 30°C (Fig. 3B). Similar stimulation was also observed with an equimolar Brr2 to Prp8-CTF ratio (25nM Brr2 and Prp8-CTF with 200nM U4/U6, data not shown). It is notable that because these reaction conditions include 8-fold excess of U4/U6 snRNA (200nm) over Brr2 (25nM), multiple turnover events per enzyme are required for the reactions to achieve completion. Prp8-CTF alone did not show any inherent helicase activity (Supplementary Fig. 2). Importantly, the unwinding activity is also dependent on a functional helicase domain in Brr2 since the Brr2-1 mutant protein was unable to unwind U4/U6 snRNAs (data not shown).

To assess the role of the RP region of Prp8-CTF in stimulating Brr2, we examined the effect of Prp8-CTF containing RP mutations on helicase activity. In particular, we highlight R2388G (Prp8-CTF-R2388G) because it demonstrated similar behavior to wild-type Prp8-CTF in yield, purity, and stability during recombinant expression and purification. Interestingly, despite the fact that Prp8-CTF-R2388G retains some affinity for Brr2 (Fig. 2B), Prp8-CTF-R2388G does not stimulate Brr2's helicase activity (Figs. 3B,C). To further characterize whether this lack of stimulation is simply due to a lack of binding between Prp8-CTF-R2388G and Brr2, we titrated the amount of Prp8-CTF-R2388G present in the helicase assay. Significantly, we observed no additional unwinding with 80 fold molar excess of Prp8-CTF-R2388G (2.0 μ M) over Brr2 (25nM) (Fig. 3D). We also examined Prp8-CTF fragments containing F2382L and R2388K in the helicase assay. Interestingly, in the case of these mutants, we observed ~40% unwinding after 60 minutes, which is approximately twice the amount of unwinding as in the Brr2 alone condition, in the presence

of 10 fold molar excess of Prp8-CTF-F2382L(250nM) or 10 or 80 fold molar excess of Prp8-CTF-R2388K (250nM, 2.0 μ M) over Brr2 (25nM) (data not shown).

Brr2's ATPase activity is modulated by Prp8-CTF

To gain insight into the mechanism of ATP-dependent unwinding activity by Brr2, we performed Brr2 ATPase assays in the presence and absence of Prp8-CTF. Using thin-layer chromatography (TLC), we measured the amount of inorganic phosphate released from 32 P gamma labeled ATP as a function of time. Importantly, the buffer conditions and temperature used in the ATPase assays are identical to those examined in the unwinding assays (shown in Fig. 3). In Figure 4A, representative TLC panels of time courses are shown. Assays performed in the absence of RNA show that Brr2 is an RNA-dependent ATPase and indicate that the background level of hydrolysis observed for each of the protein components is quite low. From these experiments, we determined the rate of ATP hydrolysis per Brr2 per minute in the presence of U4/U6 (Fig. 4B). We found that Brr2 hydrolyzes one-third as much ATP when Prp8-CTF is added to the reaction. To more fully characterize the Brr2 enzyme and Prp8-CTF co-factor relationship, we examined the RNA concentration dependence of Brr2 ATPase activity in the presence and absence of Prp8-CTF (Fig. 4C). Although the high RNA concentrations required prevent us from accurately assigning a K_m value, we did observe a significant decrease in the ATP hydrolysis rate for all RNA concentrations examined in the presence of Prp8-CTF.

Interestingly, in the presence of Prp8-CTF-R2388G, Brr2 hydrolyzes an intermediate level of ATP in comparison to reactions with Prp8-CTF or no co-factor. This suggests that Prp8-CTF-R2388G's ability to weakly bind Brr2 is able to influence Brr2's ability to hydrolyze ATP even though it shows no measurable effect on its unwinding ability. We further expanded this study to include other Prp8 RP mutations (Fig. 5). We observed a range of phenotypes for the RP mutations. Notably, F2382L, the mutation that retained the best ability to bind Brr2, showed no inhibitory effect on ATPase activity. R2388K showed the same ability to inhibit Brr2 as wild-type Prp8-CTF. Taken together with the findings of partial helicase stimulation by Prp8-CTF-R2388K and Prp8-CTF-F2382L, these findings suggest that Brr2 binding, stimulation of U4/U6 unwinding and inhibition of ATP hydrolysis are at least partially independent functions of the C terminal domain.

Discussion

Prp8 Modulates ATPase and Helicase Activities of Brr2

When base-paired with U4 snRNA in the context of the U4/U6:U5 triple snRNP, U6 snRNA is catalytically inert, a state which is promoted by the high stability of the U4/U6 duplex ($T_m = 53^\circ\text{C}$ in budding yeast)⁴³. Unwinding of U4 by the Brr2 DExD/H-box helicase is presumably precisely timed in vivo to ensure that splicing signals have been accurately recognized. Because Brr2 is a stable component of the U5 snRNP, a fundamental question is how its activity is strictly controlled to maintain fidelity. Here we have employed a minimal biochemical system to demonstrate that the C-terminal ~600 amino acids of the U5 snRNP protein Prp8 (Prp8-CTF) are required to activate Brr2 to unwind a synthetic U4/U6 duplex.

To our knowledge, this is the first report of a co-factor for a spliceosomal ATPase with a known *in vivo* target.

Importantly, the ability of the Prp8-CTF to stimulate U4/U6 unwinding is lost or substantially impaired if the protein carries *prp8* alleles responsible for retinitis pigmentosa (RP) in humans. Recent ultrastructural analyses reveal that these mutants map to a 35 amino acid unstructured tail following a highly structured alpha helix extending from a compact core comprised of a Jab1/MPN domain^{28,29}. These RP mutations inhibit the interaction of Brr2 with the Prp8-CTF to varying extents. This direct contact does not appear to be the only function of these residues, however, since increasing the concentrations of RP mutant Prp8-CTF does not lead to further helicase stimulation or ATPase activity inhibition. This suggests that binding between these mutants and Brr2 is near capacity.

An important issue for the future will be the location of the precise interaction site on Brr2. This question is particularly interesting in view of its unusual domain structure: an N-terminal canonical DEIH-box domain, and a C-terminal non-consensus DDAH motif both flanked by regions of homology to Sec63. The *brr2-1* allele lies in the N-terminal helicase domain²⁰ and inactivates the ability of the protein to unwind U4/U6 in our assay confirming that helicase activity is due to Brr2. Previous two-hybrid studies suggest that the C-terminus of Prp8 contacts a region of the second helicase motif, specifically amino acids 1301 to 1816 in human Brr2 (ref. 28), in which case the stimulatory effect on unwinding is likely to be via an allosteric mechanism.

Although the Prp8-CTF stimulates the helicase activity of Brr2, it strongly inhibits the RNA-dependent ATPase activity. Because the unregulated ATPase levels are high in this *in vitro* system, an attractive hypothesis is that one function of Prp8 is to couple ATP hydrolysis to productive unwinding events *in vivo*. Repression of a basal ATPase level by a co-factor which stimulates helicase activity has been observed previously for both a DNA helicase, DnaB, and its co-factor, SSB44 as well the RNA helicase NS3 and its co-factor NS5b⁴⁵, although the mechanisms by which the co-factor modulates the coupling of ATP hydrolysis and helicase activity remain unknown. From studies of other DExD/H-box proteins, it has been suggested that the conformational changes consequent to ATP hydrolysis are relayed via motifs III and VI to motifs IV and V, which are involved in RNA binding^{2,3,46,47}. The hypothesis that Prp8-CTF acts to couple ATPase and helicase activities is consistent with the observation that several RP alleles that are defective in unwinding also show a reduction in inhibition. Nonetheless, overall there is no consistent correlation between Brr2 interaction, stimulation of U4/U6 unwinding, and inhibition of ATP hydrolysis. Thus more work is needed to understand the relationships of these three biochemical activities to one another.

A related question is how the U4/U6 RNA substrate is recognized. To address the possibility that Prp8 stimulates the unwinding reaction by increasing Brr2's affinity for RNA, as has been seen for other DExD/H-box proteins^{48,49}, we assayed Brr2 activity as a function of U4/U6 concentration. However, we were unable to calculate a K_m for either the ATPase or helicase activities, since we could not achieve saturating levels of RNA. Presumably in the context of the spliceosome, the high local concentration of reactants obviates the need for

high intrinsic affinities. Another mechanism by which Prp8 might influence the U4/U6 unwinding function of Brr2 is by increasing the processivity of the reaction, either by direct interaction with the helicase or indirectly through the interaction with nucleic acid by trapping the single stranded nucleic acid allowing for continued unwinding of the duplex⁵⁰. In particular, such a mechanism might be warranted by the length and strength of U4/U6 stem II, the most stable duplex in the spliceosome. Interestingly, it has been suggested that Prp43's processivity is affected by Ntr1, its protein co-factor¹⁰. Experiments are in progress to further characterize the properties of the current system, including monitoring processivity per se.

Retinitis pigmentosa and splicing

Human Prp8 (RP13) is one of four splicing factors encoded by genes associated with autosomal dominant alleles of RP31,⁵¹. Prp3 (RP11) and Prp31 (RP18) are also tri-snRNP proteins, and PAP-1 (RP9) is a negative splicing regulator that binds Prp3 (refs. 52,53). Previous reports have suggested that reduced level of tri-snRNPs might be responsible for the retinal phenotype (reviewed in ref. 31), yet our results point to one more interesting possibility, namely the dysregulation of catalytic activation. In particular, Prp3 is known to bind to U4/U6 stem II⁵⁴ and Brr2-dependent unwinding disrupts this binding site, displacing Prp3 from the spliceosome. Thus the Prp3-U4/U6 interaction may likely antagonize Brr2 activity. The known sites of RP alleles in Prp3 lie outside its RNA-binding domain⁵⁵. Conceivably they identify sites of interaction that, like Prp8, modulate the activity of Brr2. Overall, this dysregulation of catalytic activation may result in broadly decreased splicing efficiency in photoreceptor cells that have high demands on the splicing machinery, resulting in cell death³¹. Conversely, the RP phenotype could be due to transcript-specific effects in these cells ^{56,57}.

A Model for Splicing Regulation

Our findings are contrary to previous genetic studies that suggested that Prp8 negatively regulates Brr2-dependent unwinding^{39,40}. In fact, we do not rule out that Prp8 may act as both a positive and negative regulator of Brr2. Indeed, our conclusion that the C-terminal region of Prp8 regulates the activation of the Brr2 helicase presupposes a mechanism by which the Prp8-Brr2 interaction is itself regulated. Recent evidence suggests several levels at which such control is likely to be effected. Staley and Sontheimer uncovered a regulatory mechanism, namely that the activity of Brr2 is negatively regulated by ubiquitination of Prp8 (ref. 58). While the site of modification on Prp8 is currently unknown, it is tempting to speculate that ubiquitin is recognized intramolecularly by interaction with the Jab1/MPN domain and that this association precludes the Brr2-Prp8-CTF interaction. Interestingly, the importance of this region is further evidenced by our preliminary result that a truncated form of Prp8-CTF containing only the last ~300 amino acids is sufficient to stimulate Brr2's helicase activity (Maeder, Kutach, Guthrie, unpublished data).

Finally, it has been shown by Staley and colleagues that Brr2 not only controls the dissociation of U4 from U6 at the beginning of the splicing cycle, but also the dissociation of U2 and U6 during spliceosome disassembly²³. In both cases, Brr2 activity is inhibited by the binding of Snu114 to GDP; indeed the spliceosomal GTPase appears to function akin to

G proteins, as a conformational switch whose activity depends on the identify of the bound nucleotide²³. While in principle the control of Brr2 activity by Snu114 could be direct, it is interesting to note that both the ATPase and the GTPase interact with the same C-terminal region of Prp8 (refs. 28,29). Since extensive genetic evidence supports a role for Snu114 in the function of Prp8 (ref. 59), an attractive hypothesis is that the nucleotide state of Snu114 controls a conformational rearrangement of Prp8, which in turn modulates the availability of the C-terminus for interaction with Brr2. Important questions that can be addressed using our minimal system include determining whether the Prp8-CTF also promotes Brr2-dependent unwinding of U2 and U6, and whether helicase activity is inhibited by Prp8 ubiquitination.

Materials and Methods

Growth assays

Strains for growth assays were created by transforming the parent strain yTB72 (*MAT a*, *lys2D*, *ura3D*, *leu2D*, *his3D*, *prp8::LYS2*, pJU169: *PRP8*, *URA3*)⁵⁹ with *PRP8*-harboring plasmids, and then subjected to selection on 5-fluoroorotic acid (5-FOA)-containing media to counter select against the *PRP8* and *URA3*-marked plasmid pJU169. The final strains were verified to be uracil auxotrophs confirming the absence of plasmid pJU169. Construction of the mutant plasmids is described in the Supplementary Methods and Supplementary Table 1. Oligonucleotides used in plasmid constructions are listed in Supplementary Table 2.

Growth assays were performed by growing yeast strains in YP + 2% (w/v) glucose liquid growth medium (YPD) overnight at 20°C. The cells were diluted to 0.1 OD₆₀₀ in liquid YPD and continued to grow for approximately 5 hours. The cultures were then diluted to 0.05 OD₆₀₀ in liquid YPD and 5-fold serially diluted. Approximately 5 µl of each strain were deposited onto replicate YPD agar medium and incubated at 20°C (five days), 30°C (2 days), or 37°C (2 days).

RNA synthesis, purification, and duplex annealing

U4 and U6 snRNA were synthesized from run-off transcriptions from pT7U4 (ref. 60) and pT7U6 (ref. 41) linearized with Sty1 and Dra1 respectively using T7 RNA polymerase. Syn U6 containing 22-116 nt of U6 snRNA was synthesized by Dharmacon. All RNAs were purified by extraction from a 6% (w/v) TBE polyacrylamide gel, phenol:chloroform extraction, and ethanol precipitation. U4/U6 duplex was made fresh from every experiment. RNAs were combined in 15mM HEPES pH 7.9 and 400 mM NaCl and heated to 75°C for 5 min. MgCl₂(10mM) was added immediately once the RNA sample was removed from heating. Samples were cooled at room temperature for 30 min. In the case of the helicase assays, duplex formation was driven by using excess synU6 (1.5 fold over U4). In the case of the ATPase assay, duplex formation was driven by using excess U4 (1.5 fold over U6). Concentrations indicated are that of the duplex.

Protein expression and purification

BL21-DE3 Rosetta cells (Novagen) were transformed with inducible T7 expression plasmids (pDEST15 (Invitrogen)). 500 ml cultures were grown at 37°C to OD₆₀₀ of 0.4, diluted with 500 ml 4°C LB medium, incubated 15 minutes at 16°C, and induced with 0.4mM IPTG. Cells were harvested after 16 hours and then frozen. To purify GST-Prp8-CTFs, frozen cell pellets were thawed in 30 ml of buffer (20mM Hepes 7.9, 300mM NaCl, 1mM BME, 0.2mM EDTA, 1mM Benzamidine, 0.05% (v/v) Np-40, 0.5mM PMSF, 2µg ml⁻¹ Pepstatin A), and disrupted by microfluidization (Microfluidics). Lysates were clarified by centrifugation, 0.45µm filtration, and adsorbed to a 0.5 ml glutathione-Sepharose (GE) resin bed. The resins were washed with five 10-column volume washes of extraction buffer, and eluted in extraction buffer plus 10 mM reduced glutathione. The eluted proteins were dialyzed twice against 2 liters of storage buffer (20mM Hepes 7.9, 100mM NaCl, 1mM BME, 0.2mM EDTA, 1mM Benzamidine, 0.01% (v/v) Np-40, and 20% (v/v) glycerol) and flash frozen in liquid N₂. Constructions of bacterial expression plasmids are described in the Supplementary Methods. Prp8-CTF and mutant concentrations were determined using calculated extinction coefficient, 122535 M⁻¹cm⁻¹.

The TAP-tagged Brr2 overexpression plasmid, pGPD-BRR2-TAP (Gift from Scott Stevens) was shuffled into yeast strain yTB105 (ref. 59). Six liters of culture were grown to OD₆₀₀ of 3 in YPD liquid medium. Cells were processed and purified following the TAP protocol³⁷ with the following exceptions: the soluble extract was supplemented to 750 mM NaCl prior to the first affinity step, and in some preparations, a ball mill was used to homogenize the frozen cell paste in place of a mortar and pestle. The final purified protein was dialyzed twice for two hours against 20mM Hepes, 100mM NaCl, 5mM BME, 1mM Benzamidine, 0.01% (v/v) Np-40, 20% (v/v) glycerol. The purified Brr2 protein retains the C-terminally fused calmodulin-binding domain. Brr2-CBP concentrations were determined using calculated extinction coefficient, 252,000 M⁻¹cm⁻¹.

GST pull down assays

Purified GST-Prp8-CTF fusions (20 pmol) were bound to glutathione-Sepharose (5 µL) for 20 min. The resins were pelleted and washed with 1 ml of binding buffer (20mM Hepes 7.9, 100mM NaCl, 1mM BME, 0.2mM EDTA, 0.01% (v/v) Np-40). The GST-Prp8-CTF-bound resins were incubated with 20 µl of 1µM Brr2 protein for 30 min with rocking. The resins were pelleted, unbound proteins aspirated, and washed twice with binding buffer (1 mL). The proteins bound to the resins were eluted by boiling in 20 µl of reducing SDS-loading dye and resolved by SDS-PAGE. Western blotting was used to detect GST-CTF and Brr2-CBP. Secondary antibodies were IR800 labeled anti-rabbit IgG that permitted detection with an Odyssey imager (Li-COR). Quantification was performed with the manufacturer's provided software for band intensity integration. Percent Brr2 bound was calculated by using the following equation: $\frac{((\text{Brr2}_{\text{mutant}}\text{-GST})/\text{Prp8-CTF}_{\text{mutant}})}{((\text{Brr2}_{\text{WT}}\text{-GST})/\text{Prp8-CTF}_{\text{WT}})} * 100$.

Helicase assays

The reaction mixtures (10µL) contained 64mM NaCl, 30 mM Tris pH7, 2.5mM MgCl₂, 1.5mM DTT, 600ng µL⁻¹ BSA, 20U RNasin (Roche) with 25 nM Brr2 and 250 nM GST-

Prp8-CTF or GST-Prp8-RP. Trace amounts of HEPES, NP-40 and EDTA are present in the reactions. U4/U6 snRNAs were incubated in the reaction mixture for 5 min at 30°C before initiating unwinding reactions with 2mM ATP. Reactions were performed at 30°C. Aliquots (1 uL) were withdrawn at time points and terminated with 19uL of stop solution (20mM EDTA, 0.2M NaCl, 1% (w/v) SDS). Time point samples (2uL) were annealed with 100fmol of Alexa Fluor 647 labeled oligo 14B (5'-AGGTATTCCAAAAATTCCC-3' which is complementary to U4 positions 140 to 158)42 for 15 min at 42°C. A zero time point sample was boiled for 2 minutes to indicate free U4. Unwound U4 and U6 were separated from duplexed U4/U6 on a 9% (w/v) native gel42. The amount of U4/U6 and U4 were quantified on an Odyssey imager (Li-Cor). The percentage unwinding was calculated as follows: (counts for free U4/(counts for free U4+counts for U4/U6))*100.

ATPase assays

All ATPase assays were performed under identical conditions to the helicase assays with the following exceptions: [γ -³²P]ATP was combined with cold ATP to make a total of 2mM in the reaction. RNA and ATP were combined separately from the remainder of the reaction mixture, and both mixtures were incubated for 5 min at 30°C. Reactions were initiated by combining RNA/ATP with the remainder of the reaction. Aliquots (1uL) were withdrawn and terminated with 2uL of 0.5M EDTA. Time-point samples (1uL) were spotted on to cellulose-PEI TLC plates (JT Baker), which was developed with 0.1M KPO₄ pH7.4 buffer. The amount of ATP hydrolyzed was quantified using a Storm phosphoimager. The percentage hydrolyzed was calculated as follows: (counts for Pi/ (counts for Pi+counts for ATP))*100 for each time point. Percentage ATP hydrolyzed was plotted against time to determine a rate of hydrolysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Scott Stevens (University of Texas, Austin) for the Brr2-TAP overexpression plasmid, Luke Rice for assistance with mass spectrometry, Joel Credle for assistance with protein purification, R. Luhrmann (Max-Planck-Institute for Biophysical Chemistry) for the gift of anti-GST antibody, and Jonathan Weissman (University of California, San Francisco) for the gift of anti-CBP antibody. We also thank John Abelson and Jeff Pleiss for helpful discussions and members of the Guthrie lab for critical reading of the manuscript. C.G. is an American Cancer Society Research Professor of Molecular Genetics. This work was supported by a grant from the National Institutes of Health to C.G. (GM21119), a National Institutes of General Medical Sciences postdoctoral fellowship to C.M. (F32GM077844), an American Cancer Society postdoctoral fellowship (PF-01-236-01-GMC) and Boyer Funds to A.K.K.

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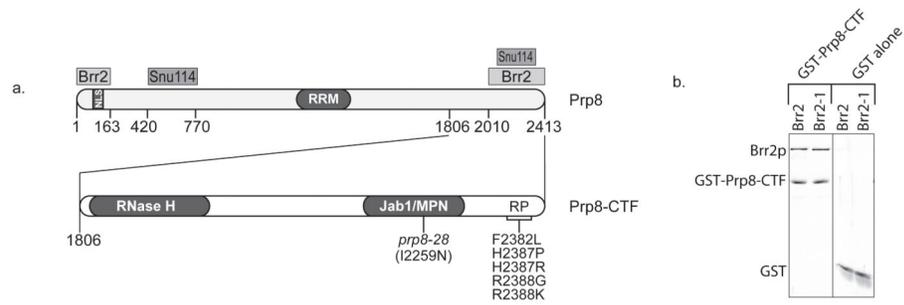
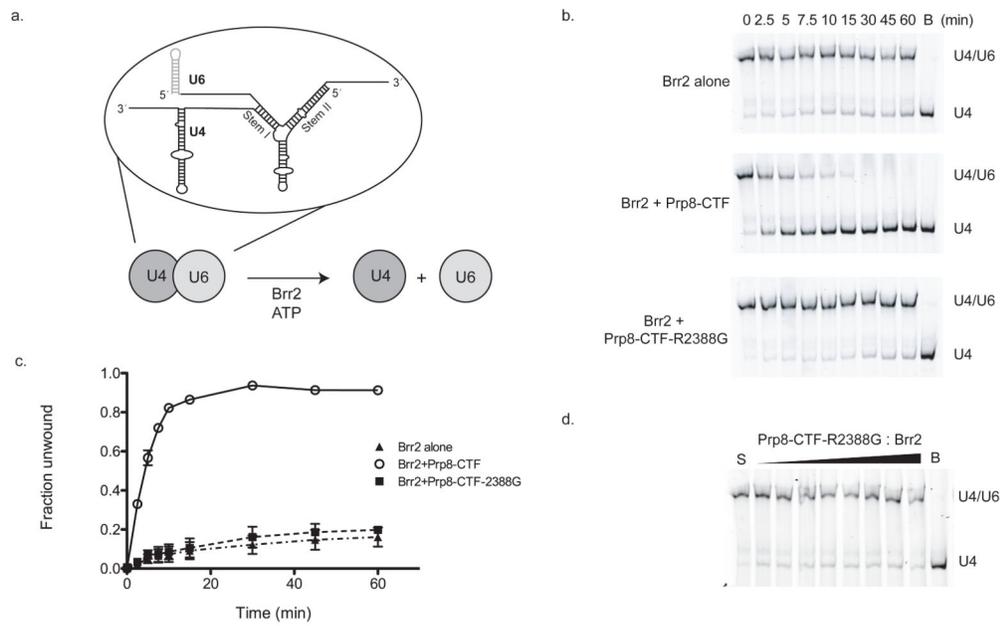


Figure 1.

A C-terminal fragment of Prp8 was able to directly interact with Brr2. **(a)** Schematic diagram of full-length yeast Prp8 and the C-terminal fragment of Prp8 (Prp8-CTF) used in this study. Yeast two-hybrid interactions with Brr2 (1-163; 2010-2413 aa)³³ and Snu114 (420-770, 2239-2335 aa)²⁷⁻²⁹ previously described are indicated as rectangles with their amino acid location noted. The NLS (96-156 aa) and RRM (1059-1151 aa)²⁶. The RNase H domain with protruding β -finger domain (1833-1990 aa)³⁴⁻³⁶ and Jab1/MPN domain (2178-2310 aa)²⁶ and RP mutants used in this study are noted on the Prp8-CTF diagram. **(b)** GST-tagged Prp8-CTF interacted equally well with CBP (calmodulin binding protein)-tagged Brr2 or CBP-tagged Brr2-1 at incubation together for 10 minutes and isolation on glutathione resin. The Western blot was performed detecting for GST and CBP.

**Figure 3.**

Unwinding of U4/U6 by Brr2 is stimulated by addition of Prp8-CTF. **(a)** Schematic diagram of the ATP-dependent Brr2-catalyzed unwinding of duplexed U4/U6 snRNAs. The grey portion of U6 snRNA is present in full-length U6 snRNA, but absent from U6syn synthesized RNA. **(b)** Native gel analysis of a time course of unwinding of U4/U6 (200nM) by Brr2 (σ, 25nM) shows that Brr2 was stimulated by the addition of Prp8-CTF (○, 250nM) but not by Prp8-CTF-R2388G (■, 250nM). B indicates an aliquot of the reaction boiled. A fluorescently labeled oligo was used to detect U4 snRNA on both U4/U6 duplex or free U4. Native gel analysis was performed on the aliquots. **(c)** Graphical representation of the unwinding of U4/U6 snRNAs in the presence of Prp8-CTF or Prp8-CTF-R2388G shown in (b). The values are average of three independent experiments ; error bars indicate s.d. **(d)** Unwinding assay of U4/U6 duplex in which increasing amounts of Prp8-R2388G are used in the reaction indicates that additional Prp8-CTF-R2388G did not result in stimulation of Brr2. Assays were performed as described in (b) with the exception that the amount of Prp8-CTF-R2388G was changed for each lane. Thirty minute time points are shown for reactions with 25nM, 50nM, 125nM, 250nM, 500nM, 750nM, 1μM, and 2μM Prp8-CTF-R2388G. S indicates the amount of U4/U6 unwind at time=0. B indicates an aliquot of the reaction boiled.

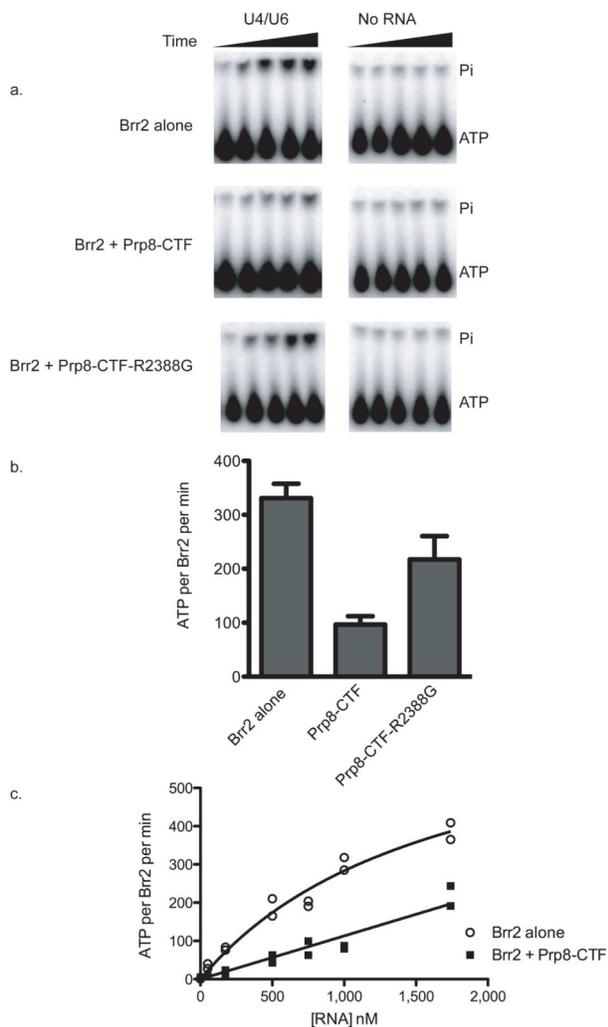


Figure 4.

ATPase activity of RNA-dependent Brr2 is inhibited by Prp8-CTF. **(a)** Time courses of ATPase activity assays for Brr2 (25nM) in the absence or presence of Prp8-CTF (250nM) or Prp8-CTF-R2388G (250nM) with 1µM U4/U6 duplex showed that Brr2's ATPase activity is inhibited by Prp8-CTF. Reaction time points were taken at 0, 4, 8, 12, and 16 minutes. **(b)** Average ATPase activities obtained from three to five independent experiments similar to (a). Error bars indicate s.e.m. A paired t-test determined that the difference between Brr2 alone and Brr2 with Prp8-CTF-R2388G is significant ($p=0.0256$). **(c)** RNA concentration dependence of Brr2's ATPase activity in the presence (■) or absence (○) of Prp8-CTF is shown. Duplicate data sets are shown, and the best fit curves are derived from the combined data sets.

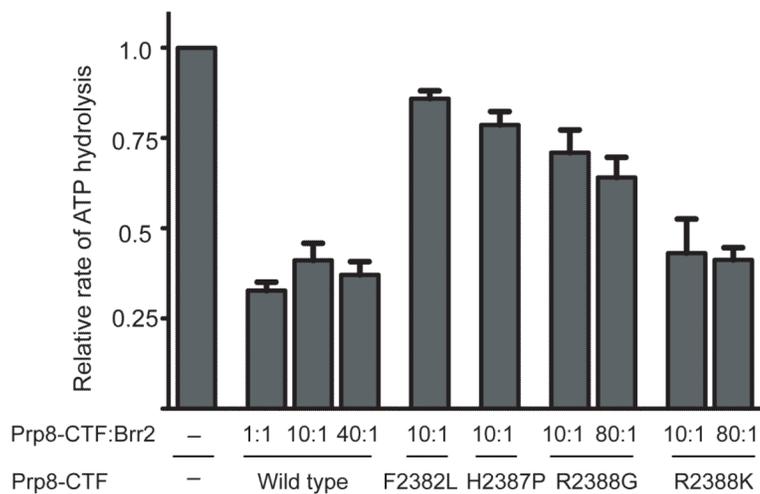


Figure 5.

ATPase activity of RNA-dependent Brr2 is affected differently by RP mutations. ATPase activity assays for Brr2 (25nM) in the presence of Prp8-CTF (250nM) or Prp8-CTF mutants (250nM) with 1 μ M U4/U6 duplex. The molar ratio of Prp8-CTF to Brr2 is indicated.

Average ATPase activities obtained from three independent experiments. Error bars indicate s.e.m.