

Recognition of the 3' splice site RNA by the U2AF heterodimer involves a dynamic population shift

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Edited by Gabriele Varani, University of Washington, Seattle, WA, and accepted by Editorial Board Member Dinshaw J. Patel September 29, 2016 (received for review April 13, 2016)

An essential early step in the assembly of human spliceosomes onto pre-mRNA involves the recognition of regulatory RNA *cis* elements in the 3' splice site by the U2 auxiliary factor (U2AF). The large (U2AF65) and small (U2AF35) subunits of the U2AF heterodimer contact the polypyrimidine tract (Py-tract) and the AG-dinucleotide, respectively. The tandem RNA recognition motif domains (RRM1,2) of U2AF65 adopt closed/inactive and open/active conformations in the free form and when bound to bona fide Py-tract RNA ligands. To investigate the molecular mechanism and dynamics of 3' splice site recognition by U2AF65 and the role of U2AF35 in the U2AF heterodimer, we have combined single-pair FRET and NMR experiments. In the absence of RNA, the RRM1,2 domain arrangement is highly dynamic on a submillisecond time scale, switching between closed and open conformations. The addition of Py-tract RNA ligands with increasing binding affinity (strength) gradually shifts the equilibrium toward an open conformation. Notably, the protein–RNA complex is rigid in the presence of a strong Py-tract but exhibits internal motion with weak Py-tracts. Surprisingly, the presence of U2AF35, whose UHM domain interacts with U2AF65 RRM1, increases the population of the open arrangement of U2AF65 RRM1,2 in the absence and presence of a weak Py-tract. These data indicate that the U2AF heterodimer promotes spliceosome assembly by a dynamic population shift toward the open conformation of U2AF65 to facilitate the recognition of weak Py-tracts at the 3' splice site. The structure and RNA binding of the heterodimer was unaffected by cancer-linked myelodysplastic syndrome mutants.

U2AF | dynamics | splicing | spFRET | NMR

During gene expression, the removal of introns is essential for translation of mature mRNA. The splicing process involves a large number of splicing factors for the correct recognition of introns (1). Whereas U1 snRNP contacts the 5' splice site, recognition of the 3' splice site involves binding of SF1/BBP to the branch point sequence (BPS) (2–5) and binding of the U2 auxiliary factor (U2AF) heterodimer to the poly-pyrimidine-tract (Py-tract) that precedes the AG dinucleotide at the intron/exon junction. Binding of U2AF to the 3' splice site during the early steps of spliceosome assembly recruits the U2 snRNP (6–9). The strength, i.e., splicing efficiency, of a 3' splice site requires recognition of the BPS, Py-tract, and the AG dinucleotide. However, of these three RNA elements, the Py-tract exhibits the largest degree of variability, and thus, weak to strong splice sites are primarily classified depending on the composition of the Py-tract (7, 10).

U2AF is a heterodimer consisting of a large (U2AF65) and a small (U2AF35) subunit. U2AF65 harbors two canonical RNA recognition motifs (RRM1,2) and an atypical C-terminal RRM domain, called the U2AF homology motif (UHM). U2AF35 has one RRM (which acts as an UHM), flanked N- and C-terminally by two CCCH-type zinc finger motifs, respectively (Fig. S14) (9, 11, 12). The U2AF heterodimer is formed by recognition of a peptide

motif, called the UHM Ligand Motif (ULM), in U2AF65 by the U2AF35 UHM domain (13, 14) and enhances RNA binding (15, 16). Recognition of the conserved AG dinucleotide at the 3' splice site requires U2AF35 (15, 17–20). U2AF65 binds to the Py-tract sequence located at the 3' splice site via its RRM domains. The Py-tract sequence is degenerate in eukaryotes (21, 22), and the efficiency of the 3' splice site recognition largely depends on the Py-tract strength, i.e., the number of uridines present in the sequence (8, 9). U2AF65 is sufficient for splicing of introns harboring strong Py-tracts in vitro, whereas U2AF35 is required for splicing of introns with weak (low-affinity) Py-tracts and is essential in vivo (7, 10, 23–25). The substantial variability in Py-tracts and plasticity in intron recognition is exploited for alternative splicing, which in eukaryotes occurs in over 60% and in humans in the majority of multiexon genes (21, 26, 27).

Recently, it was proposed that Py-tract RNA binding by U2AF65 employs a conformational selection mechanism, which involves a population shift of the arrangement of its tandem RRM domains from a closed to an open state (28). Moreover, based on a combined analysis of NMR and small angle X-ray scattering (SAXS) data, it has been shown that the unbound RRM1,2 protein adopts a range of closed and detached domain arrangements, which are not able to mediate high-affinity RNA binding (29). To understand the

Significance

The splicing of human pre-mRNAs is tightly controlled and regulated during the assembly of the spliceosome onto pre-mRNA introns. Recognition of regulatory RNA sequence motifs by splicing factors is an essential early step during spliceosome assembly. We combine single-pair FRET and NMR to show that the recognition of the 3' splice site in pre-mRNA introns by the essential heterodimeric splicing factor U2 auxiliary factor (U2AF) involves conformational dynamics and population shifts of its RNA binding domains between open and closed conformations. Unexpectedly, the small subunit U2AF35 facilitates the recognition of weak splice sites by a population shift of the RNA binding domains of U2AF65 toward the open conformation. Notably, disease-linked mutations in U2AF65 do not affect RNA or U2AF35 binding.

Author contributions: L.V.v.V., C.S.-R., H.-S.K., T.M., K.Z., L.R.W., M.S., and D.C.L. designed research; L.V.v.V., C.S.-R., H.-S.K., T.M., and K.Z. performed research; A.B. contributed new reagents/analytic tools; L.V.v.V., C.S.-R., H.-S.K., T.M., and K.Z. analyzed data; and L.V.v.V., C.S.-R., H.-S.K., M.S., and D.C.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. G.V. is a Guest Editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1605873113/-DCSupplemental.

molecular mechanisms of these conformational changes and characterize the underlying conformational dynamics induced by RNA binding, we applied single-pair Förster resonance energy transfer (spFRET). The detailed investigation of the conformational dynamics on the single-molecule level revealed that the arrangement of the U2AF65 RRM1,2 tandem domains switches between an open and closed conformation. Increasing binding affinity to Py-tract RNA ligands correlates with a population shift toward the open state. Only strong Py-tracts promote the formation of a rigid protein–RNA complex, whereas complexes with weaker, i.e., lower-affinity, RNA ligands, still show significant conformational dynamics. Most notably, the presence of the small subunit, U2AF35, shifts the U2AF65 RRM1,2 domain arrangement toward an open, active conformation already in the absence of RNA, consistent with the known requirement of U2AF35 to enhance the recognition of weak Py-tracts by U2AF.

Results

The RRM1,2 Domains of U2AF65 Are Highly Dynamic. The two RNA recognition motifs 1 and 2 (RRM1,2), connected by a flexible linker, represent the minimal RNA binding region for U2AF65 (Fig. S1) (8, 28, 30). Molecular details of the RNA recognition of the individual RRM1 and RRM2 domains have been described previously (30). Recently, it was shown that U2AF65 RRM1,2 exists in a closed conformation in its free form and an open conformation when RNA is bound (28). An enhancement of the overall RNA binding affinity with increasing Py-tract strength correlates with a population shift toward the open conformation. NMR data demonstrate that this shift involves an increased contribution of RRM1 to RNA binding (28). To investigate the role of conformational dynamics in this population shift, we used spFRET to study the intrinsic dynamics of RRM1,2 in the absence and presence of RNA. For these experiments, one residue in each of the RNA recognition motifs RRM1 and RRM2 was mutated to a cysteine residue and labeled stochastically with a donor and acceptor dye pair (Fig. 1*A* and Fig. S1*C*). The cysteine positions were designed to be in close proximity when the RRM1,2 tandem domains adopt a closed conformation and to be at maximal distance when the domains adopt an open conformation (28). To ensure that the measurements are not subject to artifacts from the labeling, we analyzed the FRET efficiency and conformational change of the protein using different fluorescent labels and tested different labeling positions (Figs. S2 and S3). The constructs were measured in solution using a confocal microscope with multiparameter fluorescence detection and pulsed interleaved excitation (MFD-PIE) (31–34).

In the spFRET efficiency histogram, the free form of RRM1,2 is found to populate a high FRET state with a mean FRET efficiency of 0.78 (Figs. 1*B* and 2*A* and Table S1). By analyzing the lifetime information of the donor fluorophore, we could determine that this FRET efficiency is an average value obtained from a highly dynamic population of molecules (Fig. 1*B*, *Left*). In burst analysis experiments, an average FRET efficiency and donor lifetime is determined for each molecule. When molecules undergo conformational dynamics during their transit time through the confocal volume (of a few milliseconds), an average single-molecule FRET efficiency is measured. The presence of dynamics on the submillisecond time scale can be visualized by plotting the intensity-determined FRET efficiency versus donor lifetime. In the presence of dynamics, the intensity-determined FRET efficiency will be a species-averaged value (Eq. S6), whereas the donor lifetime is a lifetime-weighted average (Eq. S7). This yields a deviation from the relationship observed for a static FRET species (static FRET line; Eq. S5) (32, 35, 36). In Fig. 1*B*, *Left*, all molecules clearly deviate from the static FRET line, demonstrating that the conformation of RRM1,2 is highly dynamic in the absence of RNA. By combining the lifetime information for all the single-molecule in the spFRET experiments, we observed two donor lifetimes of 0.14 and 2.15 ns for

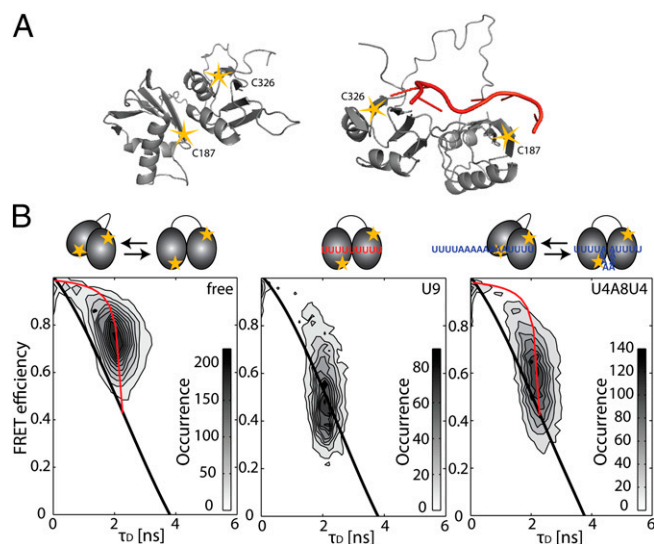


Fig. 1. Conformation and dynamics of RRM1,2 in solution. (*A*) Closed (*Left*, PDB 2YH0) and open, RNA-bound (*Right*, PDB 2YH1) structure of U2AF65 (RRM1,2) with labeling sites C187 in RRM1 and C326 in RRM2 shown as stars. (*B*) Histogram of spFRET efficiency as a function of donor lifetime of RRM1,2-Atto532-Alexa647 in the absence of RNA (9,160 molecules), in the presence of 5 μ M U9 (10,015 molecules), or in the presence of 20 μ M U4A8U4-RNA (12,083 molecules). Populations of static molecules are described by the polynomial static FRET line (Eq. S5; black line), whereas molecules undergoing conformational dynamics on the time scale of microseconds to milliseconds deviate from this line (dynamic FRET curve in Eq. S7; red line). The fully open and closed conformations were determined from lifetime fits of the data and correspond to the intersections of the dynamic FRET curve with the static FRET line (Fig. S2*B*). Representations of the open and closed conformations of the molecules are displayed schematically as a simple interpretation of the histograms.

the free form of RRM1,2, corresponding to FRET efficiencies of 0.96 and 0.43, respectively (Table S1, Fig. 1*B*, and Fig. S2*B*). Thus, we conclude that the protein undergoes conformational transitions between a closed conformation, an arrangement of the tandem domains where the donor–acceptor separation is \sim 34–40 Å and an open conformation with a distance of 64–69 Å. Estimations of the spFRET histograms for the open and closed conformation are shown in Fig. 2*D*. Using accessible volume calculations (35), we estimated the average donor–acceptor separation from the published structures of 42 Å for the closed conformation (PDB 2YH0) (28) and a distance of 61 Å for the open conformation (PDB 2YH1). The measured FRET distances and accessible volume calculations are in excellent agreement, especially when considering that the tandem RRMs represent an ensemble of closed and detached conformations with respect to each other as indicated from the NMR and SAXS data (29).

From the mean FRET efficiency and the FRET values obtained for the open and closed states from the donor lifetime data, we could estimate the fraction of time each molecule spends in the open conformation (Table S1). Free RRM1,2 molecules mainly populate the closed high FRET state (with a donor–acceptor separation of \sim 35 Å) spending an average of just \sim 1/3 of their time in the open conformation with a distance of \sim 65 Å between the fluorophores.

A linker truncation mutant RRM1,2- Δ 233–252, which was previously proposed to increase the population of the open domain arrangement (28), was investigated. This construct adopts a closed conformation with the same FRET efficiency as observed for RRM1,2, whereas the open conformation exhibits a slightly higher FRET efficiency (SI Text and Fig. S3*B*). Thus, truncation of the inner part of the linker region does not abolish the conformational dynamics of the unbound RRM1,2, whereas it promotes an open conformation that is not as extended as in the wild-type protein. At the same time, the mutant serves as an internal control

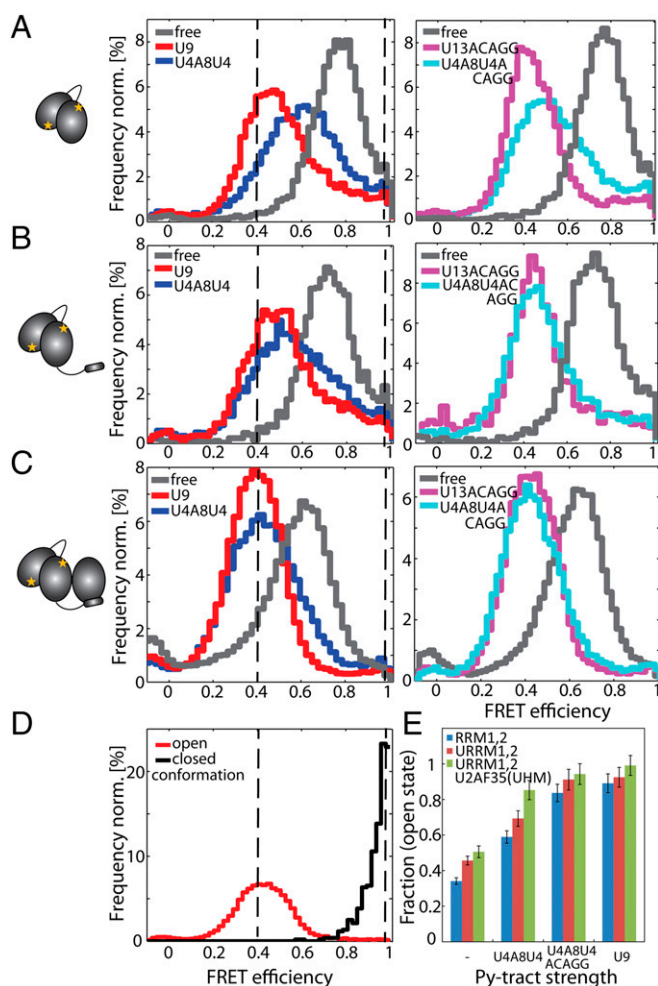


Fig. 2. Comparison of the conformations of the U2AF65-C187-C326 constructs RRM1,2, URRM1,2, and URRM1,2/U2AF35(UHM). SpFRET experiments of the constructs were performed in solution using MFD-PIE. Schematic representations of the minimal (A) RRM1,2, (B) URRM1,2, and (C) URRM1,2/U2AF35(UHM) are shown on the left. SpFRET efficiency histograms of RRM1,2 (A), URRM1,2 (B), and URRM1,2/U2AF35(UHM) (C) are displayed in the free form (gray) or in the presence of U9 (red), U4A8U4 (blue), U13ACAGG (pink), and U4A8U4ACAGG (cyan). The dashed black lines serve as guides to the eye to show the peak FRET efficiency for the open and closed conformations as obtained from D. (D) Estimation of the open and the closed state of RRM1,2. The FRET efficiency histogram of RRM1,2 molecules with a donor lifetime $\tau_D < 1$ ns (black) was selected to represent the closed conformation. The FRET efficiency histogram of U13ACAGG-bound URRM1,2/U2AF35(UHM) with a donor lifetime $\tau_D > 1$ ns (red) was selected to represent the open conformation. (E) Quantitative description of the dynamic equilibrium in a bar diagram displaying the fraction of time the different U2AF65 constructs spend in the open conformation based on the spFRET experiments of the constructs in solution (RRM1,2, blue; URRM1,2, red; URRM1,2/U2AF35(UHM), green). Errors were determined by error propagation from the values of E_1 , E_2 , and E (Table S1).

to show that another different open conformation can be adopted and recognized by the lifetime analysis and supports that observation that the open conformation observed for all full-linker constructs in the absence and presence of RNA is the same.

U2AF65 Adopts an Open State When Binding to Strong Py-Tracts.

Next, we analyzed the conformational states of the minimal RRM1,2 in the presence of RNA substrates. For the strong Py-tract (U9), the apparent FRET efficiency shifts to an average value of around 0.5 (Fig. 1B and Table S1). The spFRET histogram represents a mixture of the same open and closed conformations as observed

for the free protein (FRET efficiencies 0.97 and 0.44 for U9-bound RRM1,2) (Table S1). However, the equilibrium between the open and closed conformation of RRM1,2 is shifted significantly, with the complex spending around 90% of the time in the open conformation (Figs. 1B, Middle, and 2A). The population clearly lies on the static FRET curve indicating a stabilization of the open conformation. For a weaker Py-tract (U4A8U4), the apparent average FRET efficiency is 0.63 and the population deviates from the static FRET line (Figs. 1B, Right, and 2A). This indicates an increase in dynamics compared with the U9-bound U2AF65. Again, the spFRET data and fluorescence lifetime data reveal dynamic transitions between two states with FRET efficiencies of 0.40 ($D = 66$ Å) and 0.97 ($D = 35$ Å), i.e., the same two populations observed previously. The molecules populate the open conformation 59% of the time, whereas they were still found in the closed conformation quite frequently. Hence, the equilibrium between the open and the closed conformation of U2AF65 is influenced by the overall binding affinity for the Py-tract sequence. Py-tracts with higher affinity lead to a shift in the equilibrium by increasing the time the molecules spend in the open conformation. Consistently, purine-rich RNA ligands such as A9 and A13ACAGG that do not contain bona fide Py-tract sequences do not induce any shift in the FRET efficiency histogram or influence the conformational dynamics of RRM1,2 constructs with respect to the same protein in the absence of RNA (Fig. S2 E and F).

We investigated whether additional conformational changes are observable on a slower time scale with spFRET–total internal reflection fluorescence microscopy. Static RRM1,2-C187-C318-Atto532/Alexa647 molecules with FRET efficiencies corresponding to the conformations observed in solution-based measurements but no evidence for conformational transitions on time scales longer than 30 ms were found (Figs. S3 and S4).

Role of U2AF35 for Py-Tract Recognition by the U2AF Heterodimer.

The ULM region of U2AF65 is necessary for binding to the U2AF35 subunit and thus formation of the U2AF heterodimer. Hence, before studying the conformation of U2AF65 in the presence of U2AF35, we first characterized an extended U2AF65 RRM1,2 protein that includes the ULM region (URRM1,2) in the absence and presence of RNA (Fig. 2B and Figs. S3C and S5B). We found a similar behavior as was observed for RRM1,2 without the additional ULM region. Dynamic transitions between the open and closed conformations were observed with the same FRET efficiencies of 0.44 and 0.95 as was observed for RRM1,2 alone. We observed an overall shift of the equilibrium toward the open conformation with URRM1,2 spending 46% of the time in the open conformation in the absence of RNA (compared with 34% for RRM1,2) (Fig. 2A and B and Table S1). Binding of the strong Py-tract U9 and the weak Py-tract U4A8U4 to URRM1,2 induced a shift of the equilibrium toward the open state with the molecules now spending 92% and 69% of their time in the open conformation, respectively (Table S1). Isothermal titration experiments show that the addition of amino acids 88–147, preceding the RRM1 domain, increases the affinity of URRM1,2 sixfold compared with RRM1,2 alone (Table S2). We could show that this increased affinity is associated with faster binding rates (Table S2). An increasingly open conformation is thus accompanied by an increase in the binding kinetics.

Next, we investigated the role of U2AF35 in Py-tract recognition at the 3' splice site. The U2AF35 UHM domain mediates protein–protein interactions (13, 14) and folds upon binding to a UHM Ligand Motif (ULM) peptide sequence in U2AF65 (11) but does not directly contribute to the specific recognition of the AG-dinucleotide by U2AF35 (37). Instead, two conserved zinc finger domains flanking the UHM are expected to mediate the specific recognition at the 3' splice site (20). To understand the contribution of the U2AF35 UHM domain to Py-tract recognition by U2AF65, we studied the RRM1,2 domain arrangements

in the context of a minimal U2AF heterodimer, which has been used in previous studies (17, 19). The minimal U2AF heterodimer comprises the U2AF65 ULM and the RRM1,2 domains bound to the U2AF35 UHM domain (URRM1,2/U2AF35(UHM)). We measured the conformation of the RRM1,2 domains within the minimal heterodimer in the absence and presence of RNA. As before, we observed dynamics between the same open and closed conformations with the heterodimer spending even more time in the open conformation (51%) with respect to RRM1,2 (34%) and URRM1,2 (46%) (Table S1). Correspondingly, the affinity of the URRM1,2/U2AF35(UHM) heterodimer ($K_D = 150$ nM) for the strong Py-tract U9 is higher than for RRM1,2 ($K_D = 1.34$ μ M) and URRM1,2 ($K_D = 220$ nM) (Table S2). The shift of the equilibrium in the presence of U2AF35(UHM) is more strongly pronounced in the presence of weak Py-tract RNA, 99% in the open conformation for the strong U9 Py-tract versus 90% without U2AF35 and 85% for the weaker ligand (U4A8U4) versus 59% when the small subunit is absent (Table S1). The presence of U2AF35 has a large effect in shifting RRM1,2 to the open conformation when the weak Py tract is bound (U4A8U4). U2AF35 also shifts RRM1,2 more to the open state when a strong Py-tract is bound, but this effect is less dramatic because the complex already exists predominantly in the open conformation. In either case, the likelihood of the heterodimer to be found in the open state is significantly increased compared with RRM1,2 or URRM1,2.

To explore potential contributions of additional RNA sequences present in the 3' splice site on the RRM1,2 conformational equilibrium, we studied the interaction with an RNA comprising a strong Py-tract followed by an AG splice site, U13ACAGG. U2AF65 RRM1,2 shows the same open and closed conformations as in the presence of the shorter Py-tract U9 (Fig. 24), consistent with the expectation that RRM1,2 specifically recognizes the Py-tract region of the RNA. The RRM1,2 proteins spend around 96% of the time in the open conformation. Upon binding to a weak Py-tract followed by the AG splice site, U4A8U4ACAGG, the equilibrium is shifted toward the open state (84%) compared with the binding of the weak Py-tract without the extended AG sequence, U4A8U4 (59%) (Fig. 24 and Table S1). Presumably, additional, nonspecific contacts involving the additional nucleotides following the weak Py-tract can induce a population shift toward the open state.

We also observe an increased stabilization of the open conformation upon binding of a weak Py-tract to URRM1,2 (Fig. 2B and Fig. S3C and Table S1). In the presence of U4A8U4ACAGG, URRM1,2 proteins spend 91% of their time in the open conformation compared with 69% for U4A8U4. This may correlate with the higher overall binding affinity of URRM1,2 for U4A8U4ACAGG than for U4A8U4. Additional contacts between U2AF65 and the additional nucleotides present downstream of the Py-tract thus lead to a higher probability of the RRM domains to be found in the open conformation. Additional chemical shift perturbations in the NMR data also revealed stabilizing RNA contacts for a few residues preceding the first β 1-strand in RRM1 (Fig. S6A), consistent with a recent crystal structure of an extended RRM1,2 protein bound to a strong Py-tract (38). However, the NMR titration data also demonstrate that no additional contacts are observed for residues preceding this N-terminal region of RRM1.

U2AF35 Enhances Py-Tract Recognition by a Dynamic Population Shift. Surprisingly, in the minimal heterodimer, the population of the open conformation of RRM1,2 is substantially increased even in the absence of RNA, going from 34% for RRM1,2 and 46% for URRM1,2 to 51% in the U2AF heterodimer (Fig. 2 and Fig. S3 C and D). This indicates that the presence of the U2AF35 UHM domain enhances the open conformation of U2AF65 RRM1,2. Consistent with the increased fraction of open conformations in the unbound U2AF heterodimer, binding of a weak Py-tract U4A8U4ACAGG

shows a dramatic population shift toward the open conformation similar to that measured in the presence of U9 (94% for U4A8U4ACAGG versus 99% for U9) (Fig. 2C and Table S1). A control experiment of RRM1,2 without the ULM binding domain in the presence of U2AF35(UHM) and U4A8U4ACAGG could not induce this stabilization of the open conformation (Fig. S5A). Thus, the presence of the U2AF35 UHM domain, when stably bound in the U2AF heterodimer, leads to a population shift of the RRM1,2 domains toward the open conformation. Thereby, the heterodimer promotes an open conformation that enables efficient binding even of weak Py-tract RNA ligands (Table S1 and Fig. 2 C and E).

An Interface in the U2AF Heterodimer Mediates the Population Shift.

To characterize the structural mechanisms of how U2AF35 leads to a population shift toward the open conformation of RRM1,2, we studied the domain arrangements in the minimal U2AF heterodimer [URRM1,2/U2AF35(UHM)] using NMR spectroscopy. We determined local tumbling correlation times from 15 N NMR relaxation data for the free U2AF heterodimer and when bound to a strong Py-tract AG RNA (U13ACAGG). These data indicate that, in the absence of RNA, the U2AF65 ULM, RRM1, and the U2AF35 UHM domains tumble together, whereas the U2AF65 RRM2 domain exhibits higher mobility (Fig. 3A). This suggests that the RRM2 may be partially detached from RRM1. In contrast, upon formation of the U2AF/RNA complex, all domains in both subunits tumble together, indicating the formation of a compact and rigid protein/RNA complex (Fig. 3 B and C).

The relative arrangement of the domains in the U2AF heterodimer/RNA complex was determined by paramagnetic relaxation enhancement (PRE) data with single nitroxyl spin labels attached to specific sites in U2AF65 RRM1, RRM2, and U2AF35 UHM (Fig. 3 D and F and Fig. S6). Notably, SL155 shows the same PRE effects for RRM1,2 in the context of the heterodimer bound to the extended strong Py-tract as observed for RRM1,2 alone bound to a strong Py-tract (Fig. S6C). This is fully consistent with the FRET data, which indicate that the same open conformation is adopted upon binding to strong Py-tracts. A number of spin labels attached to RRM1 or UHM show strong intermolecular PRE effects between these two domains (Fig. 3 and Fig. S6). These data demonstrate that the U2AF35 UHM domain shares a binding interface with the U2AF65 RRM1 domain in addition to the known recognition of the U2AF65 ULM peptide on the helical face of the UHM domain. A structural model for the domain arrangements in the U2AF heterodimer when bound to a strong 3' splice site Py-tract AG RNA is shown in Fig. 3F. Notably, the same interaction is already present in the absence of RNA (Fig. S6D). Thus, the RRM1/UHM domain interaction destabilizes the closed conformation of the U2AF65 RRM1,2 tandem domains and thereby promotes the population shift toward the open domain arrangement and thus contributes to an increased RNA affinity even for weak Py-tracts.

Disease-Linked Mutations in U2AF65 Do Not Affect the Structure and RNA Binding of the U2AF Heterodimer.

Yoshida et al. (39) described point mutations in several components of the splicing machinery leading to myelodysplastic syndromes. Here we investigated the effect of the confirmed somatic mutations L187V and M144I. To analyze whether these mutations could affect the structure of U2AF65 or the conformation of the heterodimer [by modulating the interface with U2AF35(UHM)], we compared NMR spectra of 15 N-labeled U2AF65 in the wild-type heterodimer with URRM1,2-M144I/U2AF35(UHM) and URRM1,2-L187V/U2AF35(UHM). Analysis of the chemical shift differences shows mainly local effects in the vicinity of the mutation (Fig. 4). This demonstrates that the structure of U2AF65 RRM1,2 and the formation of the U2AF heterodimer is not affected by these disease mutations. We then tested if the mutations could affect RNA binding by the

Expression and Purification of Recombinant Proteins. U2AF65 mutants were expressed in *Escherichia coli* BL21(DE3) and purified as described in Mackereith et al. (28). For the U2AF heterodimer, URRM1,2 and U2AF35(38-152) were expressed and purified separately, incubated at equimolar ratios, and purified as a complex by size-exclusion chromatography as described in Kellenberger et al. (11).

Single-Pair FRET Measurements. Single-pair FRET measurements were performed on a custom-build confocal microscope using multiparameter fluorescence detection with pulsed interleaved excitation (SI Text) (32). Proteins were site-specifically labeled at cysteines and diluted to concentrations of 20–50 pM in 20 mM potassium phosphate (pH 6.5), 50 mM NaCl. Measurements were performed with proteins in their free form or mixed with 5 μ M of U9, U13ACAGG, A9, or A13ACAGG or 20 μ M of U4A8U4, U4A8U4ACAGG, or U4A8U5ACAGG.

NMR Spectroscopy. All NMR samples contained protein concentrations of 0.1–0.5 mM in 20 mM sodium phosphate (pH 6.5), 50 mM NaCl, 2 mM DTT, and 5–10% (vol/vol) 2 H₂O. Spectra were recorded at 295 K and analyzed using Sparky 3 in combination with previously published assignments (28).

ACKNOWLEDGMENTS. We thank W. Kügel for providing the burst analysis software, and W. Schimpf and J. Valcarcel for valuable discussions. We are grateful to Y. Zhang for constructs used in preliminary experiments and G. Demiraslan for technical assistance. We gratefully acknowledge the financial support of the Deutsche Forschungsgemeinschaft through Grants SFB1035 (Projects A11, B03) and GRK1721, and support from the Ludwig-Maximilians-Universität through the Center for NanoScience and the BioImaging Network. T.M. was supported by the Bavarian Ministry of Sciences, Research and the Arts (Bavarian Molecular Biosystems Research Network), the German Research Foundation (Emmy Noether Program MA 5703/1-1), and the Austrian Science Fund (FWF; Grants P28854 and DK-MCD W1226). L.R.V. acknowledges a Long-Term EMBO postdoctoral fellowship (Grant ALTF 1520-2011).

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