

Insight into the structural basis for dual nucleic acid - recognition by the Scaffold Attachment Factor B2 protein

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Abstract:

The family of scaffold attachment factor B (SAFB) proteins comprises three members and was first identified as binders of the nuclear matrix/scaffold. Over the past two decades, SAFBs were shown to act in DNA repair, mRNA/(l)ncRNA processing, and as part of protein complexes with chromatin-modifying enzymes. SAFB proteins are approximately-100-kDa-sized dual nucleic acid-binding proteins with dedicated domains in an otherwise largely unstructured context, but whether and how they discriminate DNA- and RNA-binding has remained enigmatic. We here provide the SAFB2 DNA- and RNA-binding SAP and RRM domains in their functional boundaries and use solution NMR spectroscopy to ascribe DNA- and RNA-binding functions. We give insight into their target nucleic acid preferences and map the interfaces with respective nucleic acids on sparse data-derived SAP and RRM domain structures. Further, we provide evidence that the SAP domain exhibits intra-domain dynamics and a potential tendency to dimerise, which may expand its specifically targeted DNA sequence range. Our data provide a first molecular basis of and a starting point towards deciphering DNA- and RNA-binding functions of SAFB2 on the molecular level and serve a basis for understanding its localization to specific regions of chromatin and its involvement in the processing of specific RNA species.

Keywords:

Scaffold attachment factor proteins; nuclear matrix; Nuclear magnetic resonance spectroscopy; SAP domain; RRM domain; dual nucleic acid-binding; chromatin; RNA-processing; protein dynamics

1. Introduction

Recent studies have revealed a large number of canonical RNA-binding proteins (RBP), now known to be involved in chromatin remodeling and transcriptional regulation [1]. Similarly, cell-wide proteomics-based studies have suggested numerous DNA-binding proteins (DBP) to carry out functional roles in the processing of RNAs. In a rough classification, DRBPs can perform mutually exclusive or simultaneous DNA-/RNA-binding [2]. Interestingly, they can act as binders of the same gene on the DNA and transcript level, e.g. known for SMAD4 [3] and NF90 [4,5]. While for few proteins a dual nucleic acid-binding capability using identical domains was proven on the structural level [6-9], we have only limited insight how simultaneous DNA/RNA-binding is achieved.

A canonical class of DRBPs is represented by the group of scaffold attachment factor (SAF) proteins. Type A SAF (SAFA, SAF-A), also known as hnRNP U, had been identified as RNA-binder and interactor with chromatin in the early 1990s almost simultaneously [10,11]. The family of type B SAF proteins (SAFB) comprises three members: SAFB1, SAFB2 and the SAFB-like transcriptional modulator SLTM. Similar to SAFA, they were first identified as binders of the nuclear matrix [12], where they interact with AT-rich DNA within scaffold/matrix attachment regions (S/MARs). Very early, SAFBs were also found to locate to subnuclear structures together with the protein HSF1 upon heat stress [13], which is in line with recent findings of SAFBs in the context of heat stress recovery [14]. Meanwhile SAFBs are shown to act in DNA repair, transcriptional control, mRNA/(l)ncRNA processing, splicing regulation, and as part of complexes with chromatin-modifying enzymes [15-22]. Particularly, SAFB2 was described as a factor to assist the processing of suboptimal stem-loop structures in miRNA maturation [23], while it abundantly locates to promoter regions and e.g. interacts with the transcriptional regulator hnRNPUL1 [23]. Very recently, it was described in direct interactions with class-I transposable elements, where it apparently fulfills a function in their methylation [24].

SAFBs have been correlated with anti-tumor activity based on transcriptional repression [19,25], in particular their inhibitory interaction with transcription factors and nuclear receptors [22,26,27]. Human SAFB proteins are between 915 and 1034 amino acids long, and they all comprise highly-conserved SAP and RRM domains, primarily known for DNA- and RNA-binding, respectively, in their N-terminal half (Figure 1A-C). In addition, they contain a coiled-coil (CC)- and the little-investigated intrinsically disordered Glu-/Arg-rich (RE), Arg-/Gly-rich (RG/RGG) and Gly-rich regions (IDRs) in their C-terminal half. Those are primarily thought to confer protein-protein interactions (PPI), e.g. in oligomerization, interaction with DROSHA and SR proteins [15]. The CC-region comprises a central role in PPIs of SAFBs with the protein ERH (*enhancer of rudimentary homology*) [15], which has a redundant function with SAFB2 [28]. The RGG part -based on its close relation with SAFA [10]- possibly functions in RNA-engagement. For the RE-stretch literature suggests a role in the formation of nuclear stress bodies, foci of phase separation [29], in which SAFBs stabilize heterochromatin via interaction with transcripts [17]. Collectively, these functions involve the interaction with DNA and/or RNAs and thus, require the presence of the dedicated nucleic acid-binding domains (NBDs) to confer specificity.

Precise target sequences of SAFB NBDs have remained unknown, and so has a clear proof for the unambiguous NA type preference for either of them. The ENCODE database lists a C(A/C)CCc motif derived from an RNA Bind-n-Seq (RBnS) experiment for full-length SAFB2 [30,31]. Such a C-rich short motif may well be bound by the RRM-domain [32], specialized in ssRNA recognition in literally all RNA-processes organized by RBPs [33]. The SAP domains, in turn, have an important role in maintaining chromatin structure, while their concrete target sequences are unknown. For SAFB2, there is no particular indication of a consensus target motif apart from its early categorization as an AT-rich DNA-binder [12]. Other than for SAFB2, available ChIP-seq data [34] and follow-up studies have revealed precise binding sites with DNA promoter regions for SAFB1 [21], but a consensus motif was not derived. Of importance, SAFBs were

shown to multimerize [23], presumably and with functional relevance, through their CC regions [23], which may support simultaneous functions and modulate target NA specificity through avidity.

As of now, we lack any experimental structural information from all three SAFBs, i.e. their atom-resolved basis for specific interaction with DNAs, RNAs and proteins. Attempts toward structural insight into the dual nucleic acid-binding of SAFB2, i.e. for example RNA processing and the simultaneous anchoring to the nuclear matrix/transcription machinery interacting with DNA, have not been undertaken to date.

We here set out to examine the basis of dual nucleic acid-recognition by human SAFB2 via the dedicated NBDs. We used NMR spectroscopy to identify domain boundaries of its SAP and RRM domains and their stoichiometries, and monitored their preferences for DNA and RNA motifs. Comprehensive NMR assignments facilitate mapping of binding sites with NAs to high-confidence structural models derived from sparse data and confirmed by mutations. Surprisingly, NMR data and additional biophysical experiments suggest the SAP domain to exhibit intra-domain dynamics and possibly to occur in a minor dimer conformation. Altogether, our findings represent a first important building block towards a comprehensive functional picture of SAFB D/RNPs suggested by their plastic compositions.

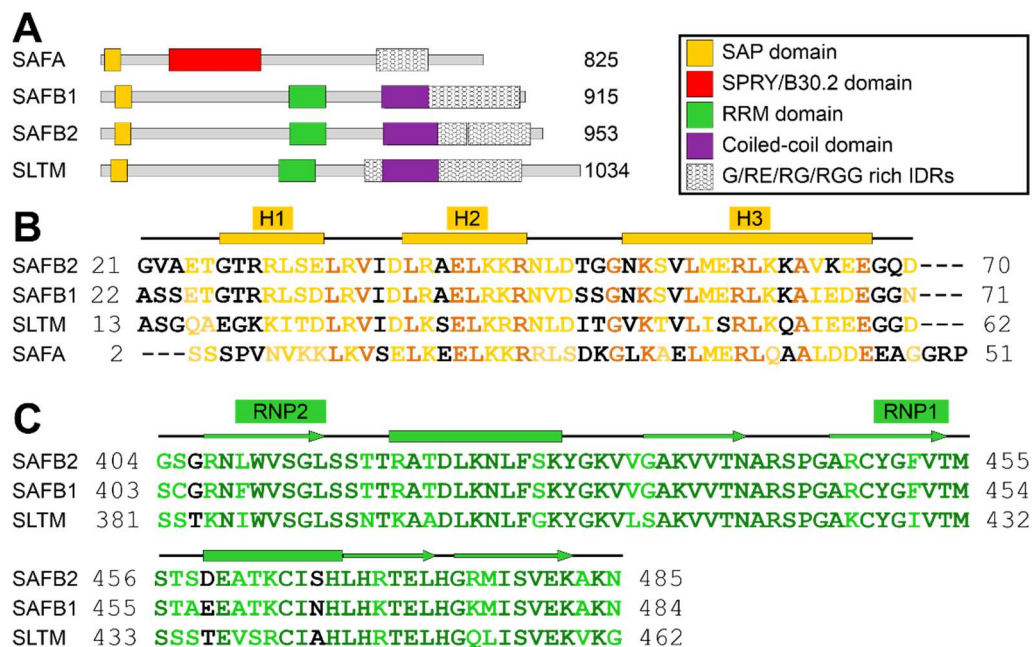


Figure 1. Scaffold attachment factor proteins (SAF) type A (hnRNP U) and B (SAFB1, SAFB2 and SLTM). (A) Domain organizations of human SAFA, SAFB1, SAFB2 and SLTM as annotated in Uniprot [35] (IDs: Q00839, Q15424, Q14151, Q9NWH9). **(B)** Sequence alignment of the SAP domain (based on domain boundaries of the SAFB2 SAP defined in this study) with residues colored-coded by conservation amongst the four proteins according to Clustal Omega [36] (black – no conservation, light orange – weak-to-strong conservation, dark orange – full conservation). Above, secondary structure elements derived from the SCS-analysis for SAFB2 SAP in Figure 2 are given, with rectangles indicating α -helices. The helix-loop-helix DNA-binding motif spans helices 2 (H2) and 3 (H3). **(C)** Sequence alignment of the SAFB RRM domains (boundaries are based on those of the SAFB2 RRM domain defined in this study) with residues colored according to the degree of conservation as by Clustal Omega (black – no conservation, light green - weak-to-strong conservation, dark green – full conservation). Above, the two conserved RNA-binding motifs (RNP) and secondary structure elements derived from the SCS-analysis for SAFB2 RRM in Figure 3 are given, with rectangles indicating α -helices and arrows indicating β -strands, respectively.

2. Results

2.1. Definition of SAFB2 SAP domain

SAP domains are described as 35-residue helix-loop-helix DNA-binding motifs (Figure 1B) [37]. The canonical SAP domain can be extended by flanking regions, contributing to affinity and/or specificity towards DNA targets [38]. Here, we cloned and expressed a 5.87 kDa region (residues 21 to 70) of human SAFB2 expected to comprise the canonical SAP domain and potential extensions (Supplementary Figure 1). The quality of purified SAP was confirmed by Nuclear Magnetic Resonance (NMR) spectroscopy. The ^1H - ^{15}N HSQC spectrum shows a well-dispersed peak pattern (Figure 2A), suggesting a folded protein species. This allowed a straightforward and complete backbone assignment of the natural sequence (Gly21 to Asp70) and the three artificial N-terminal residues (numbered Gly18-Ala19-Met20). As an exception, the amide of (non-native) Gly18 is line-broadened caused by exchange with the solvent. Additionally, we were able to assign the sidechain amides of Asn46, Asn52 and Gln69. All backbone carbons were assigned except for the Gly18 $\text{C}\alpha$. Based on $\text{C}\alpha/\text{C}\beta$ resonances we calculated carbon secondary chemical shifts (SCS) of SAFB2 SAP, relative to random coil values (Figure 2B) [39]. Three consecutive residues with significant positive shifts (i.e. >1) were the basis for defining α -helices [40,41], for which we found the expected helix-loop-helix motif plus an additional one N-terminal to it. Thus, the SCS revealed SAFB2 SAP to constitute the canonical SAP fold. Interestingly, the SCS analysis also suggested the two inter-helical stretches to adopt medium and low β -strand propensity, respectively (defined as four consecutive amides with significant negative shifts, i.e. <1). We next measured the steady-state NOEs of NH resonances to visualize internal ns-ps-dynamics within the SAP domain (Figure 2B). N- (18 to 29) and C-terminal residues (68 to 70) of the protein displayed high flexibility in solution, as indicated by hetNOE values below 0.5. This argues for a correctly defined length of the initial SAP domain construct suggested in this work. Residues 30 to 67 display hetNOE values above 0.65, with the exception of the stretch from 34 to 36. The increased flexibility of this stretch refutes a β -strand character, which had been indicated by the respective SCS values. Further, the loop connecting the two helices of the HLH motif (residues 46 to 51) exhibits rigidity -as evident from the high hetNOE values- in line with its subordinate β -strand propensity.

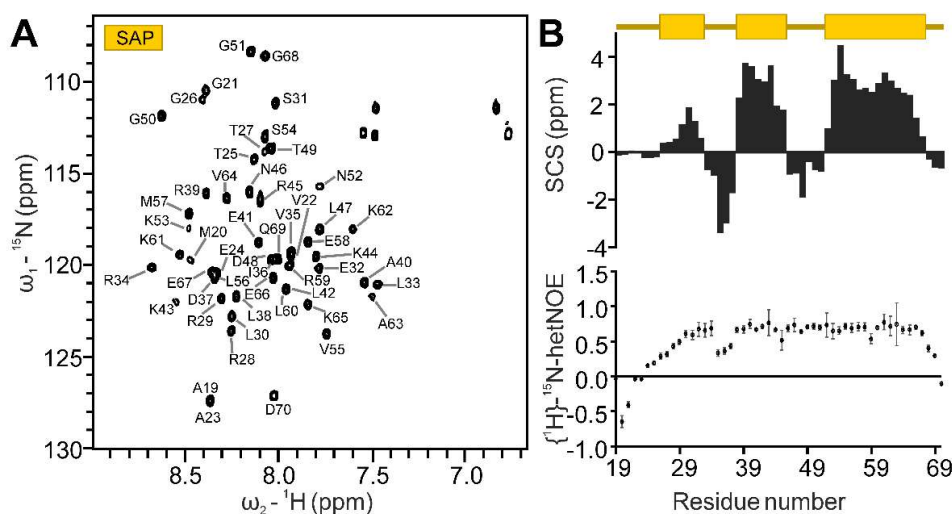


Figure 2. Resonance assignment of the SAFB2 SAP domain. (A) ^1H - ^{15}N HSQC of the SAP domain amide groups. (B) Combined $\text{C}\alpha/\text{C}\beta$ carbon secondary chemical shifts (SCS) (upper panel, as suggested by [42] – rectangles indicating α -helices) and the $\{^1\text{H}\}$ - ^{15}N heteronuclear NOE values (lower panel) of SAFB2 SAP plotted against the residue number. Errors are calculated from the program *CCPNMR Analysis*.

2.2. Definition of the SAFB2 RRM domain boundaries

A canonical RRM domain contains two conserved RNA-binding motifs (RNP1 and RNP2), embedded within a β -sheet surface (Figure 1C). Each motif is typically composed of aromatic residues that mediate base-stacking with target RNA molecules. The SAFB2 RRM domain was designed to comprise residues Gly404 to Asn485, in analogy to the human RBM5 RRM domain (PDB 2LKZ, [43]). The quality of the purified 9.13 kDa RRM domain (Supplementary Figure 1) was confirmed by NMR spectroscopy. Like for SAP, RRM peaks in the ^1H - ^{15}N HSQC spectrum were well-dispersed (Figure 3A). The backbone assignment is complete for all the 81 non-proline residues' amides in the natural sequence as well as two of the artificial N-terminal residues (numbered Gly401-Ala402-Met403 for convenience). Consequently, backbone carbons were assigned to completeness, and we also assigned sidechain amides of Trp410, Asn425, Asn441, and Asn485. SCS of SAFB2 RRM revealed the typical RRM fold: $\beta\alpha\beta\beta\alpha\beta\beta$ (Figure 3B) [32]. The $\{^1\text{H}\}^{15}\text{N}$ heteronuclear NOE spectra revealed the RRM termini to be flexible and thus the domain boundaries not to disrupt the structure. A flexible loop between $\beta 2$ and $\beta 3$ confirmed the RRM to adopt the canonical RRM fold.

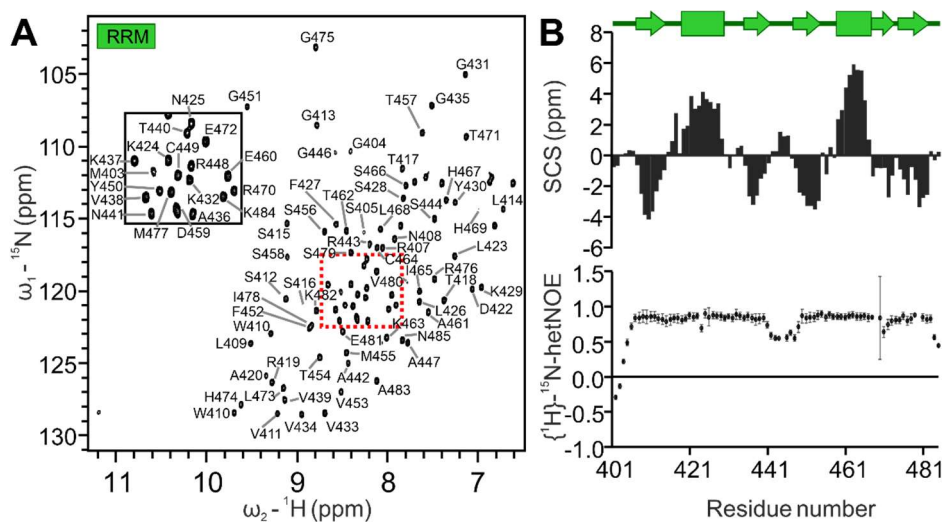


Figure 3. Resonance assignment of the SAFB2 RRM domain. (A) ^1H - ^{15}N HSQC of the RRM domain amide groups. Assignments of peaks in the red dashed-lined box are shown in the inset zoom-in. (B) Combined $\text{C}\alpha/\text{C}\beta$ carbon secondary chemical shifts (SCS) (upper panel, as suggested by [42] - rectangles indicating α -helices, arrows indicating β -strands) and the $\{^1\text{H}\}^{15}\text{N}$ heteronuclear NOE values (lower panel) of the SAFB2 RRM plotted against residue numbers. Errors are calculated from the program *CCPNMR Analysis*.

2.3. Sparse NMR data-derived structural models of SAP and RRM domains

NMR chemical shifts carry valuable structural information about the containing protein domains [44]. To gain deeper structural insight in the two SAFB2 domains we next used domains as predicted by AlphaFold [45] and compared them to NMR-derived structures based on sparse data (i.e. the obtained assignments of chemical shifts as given in 2.1 and 2.2). We used the CS-Rosetta algorithm [46] and obtained well-converging ensembles for both the RRM and the SAP domains (Supplementary Figure 2A and B). Of note, both domains are in very good agreement with the models predicted by AlphaFold (Supplementary Figure 2D and F). The RRM forms a canonical domain-type following the β - α - β - β - α - β - β domain architecture. In support of Figure 3, the folded region comprises residues Gly408 to Lys482, and a comparison with selected RRMs derived from a DALI search [47] confirms the canonical nature of the SAFB2 RRM (Supplementary Figure 2C) [48].

Both AlphaFold and CS-Rosetta reveal a compact fold of SAP for the region between R29 and G68 (Supplementary Figure 2B), which is line with hetNOE data in Figure 2. The SAP domain reveals the anticipated helix-loop-helix core, a highly conserved structural motif within SAP domains, and fully converging for SAFB2 (Supplementary Figure 2B). Importantly, this motif is present in literally all types of SAP domains, irrespective of their functional context and DNA preferences (Supplementary Figure 2E-H) as suggested by the DALI-based structure alignment with the first three selected SAP domains according to the internal score [38,49,50]. Noteworthy, both the SAFB2 SAP structure suggested by CS-Rosetta as well as the AlphaFold model show a short N-terminal helical extension (Arg29-Leu33), which suggests a more occluded hydrophobic core of the domain. Apart from that, the region N-terminal to residue 29 is clearly unstructured and a truncated construct (26-70) reveals the same core domain fold compared to the main construct (21-70) as evidenced by comparison of HSQC spectra (Supplementary Figure 3). In contrast the C-terminal boundary as used in our construct suggests to be embedded in a yet-structured context despite the lack of clear secondary structure beyond residue Gly68.

Table 1: Nucleic acid sequences used to examine the binding preferences of the SAFB2 RRM and SAP domains in this study.

Name	Sequence 5' → 3'	Usage
DNA Oligos used for binding studies		
AT _{9mer_fw}	CAATATAAC	Hybridized to AT _{9mer} , for NMR titration.
AT _{9mer_rev}	GTTATATTG	
AT _{16mer_fw}	GCGCACAAATATAACGC	Hybridized to AT _{16mer} , for NMR titration.
AT _{16mer_rev}	GCGTTATATTGTGCGC	
GC _{16mer_fw}	CGCCCCGCGCCCCGCG	Hybridized to GC _{16mer} , for NMR titration.
GC _{16mer_rev}	CGCGGGCGCGGGGGCG	
AT _{13mer_fw}	[FAM]-GCAATAAATACG	Hybridized to AT _{13mer} , for fluorescent EMSAs.
AT _{13mer_rev}	CGTATTTATTGC	
GC _{13mer_fw}	[FAM]-GCCCCGCGCCCCG	Hybridized to GC _{13mer} , for fluorescent EMSAs.
GC _{13mer_rev}	CGGGCGCGGGGGCG	
RNAs used for binding studies		
En+	UACACC	ENCODE ^(a) consensus
En-	CGGACU	ENCODE ^(a) control
U ₇	UUUUUUU	Poly-N U-rich
A ₇	AAAAAAA	Poly-N A-rich
C ₇	CCCCCCC	Poly-N C-rich
G ₇	GGGGGGG	Poly-N G-rich
Bulge	GGAUUUUUAUGGGGCACGGACAACCAUAUCCUGAU	Multi-characteristic model RNA

^(a) Motif based on an RBnS-derived entry available in ENCODE [30]

2.4. Target RNA-preferences of RRM and mapping of binding site

In order to determine target RNA preferences for the SAFB2 RRM, we compared CSPs induced by various RNAs (Table 1). Besides a set of Poly-N 7mer RNAs (U₇, A₇, C₇ and G₇) we included a 6-mer RNA with the sequence 5'-UACACC-3' (termed En+), based on the available ENCODE entry (accession number ENCSR558RBK) [30]. The RBnS (*RNA-Bind-and-Seq*) derived motif was obtained for pulldown against fl-SAFB2 and might reflect the RNA preferences of its RBD. As a control for the ENCODE motif, a 6-mer with a purine-core motif - 5'-CGGACU-3' (termed En-) - was tested.

In line with RNA-binding preferences described for a number of RRMs [51,52], we found C₇ and A₇ to induce significant CSPs of RNP residues (Figure 4A). U₇ was the least preferred motif. G₇ could not be

analyzed quantitatively, due to severe precipitation upon addition to RNA, likely induced by G-quadruplex formation. Interestingly, the RBNS-derived En+ sequence as well as the En- control resulted in similar, and in sum lower CSPs compared to A₇ and C₇ (Figure 4B). This, however, does not exclude a relevance of this motif in a genomic context. Of note, trajectories of CSPs were the same for all tested RNAs and only the maximum values varied in dependence of the RNA. This clearly argues for a shared interaction mechanism, but distinct affinities.

We next aimed at determining the binding sites of respective nucleic acids to the RRM domain. To this end, we quantified CSPs and mapped significantly affected residues on the lowest-energy CS-Rosetta structure of the RRM domain (Figure 4C). As expected, we found residues most perturbed that form the two classical RNP motifs of the domain, as well as two C-terminal residues extending the β -sheet surface. This indicates the canonical RRM interaction with RNA.

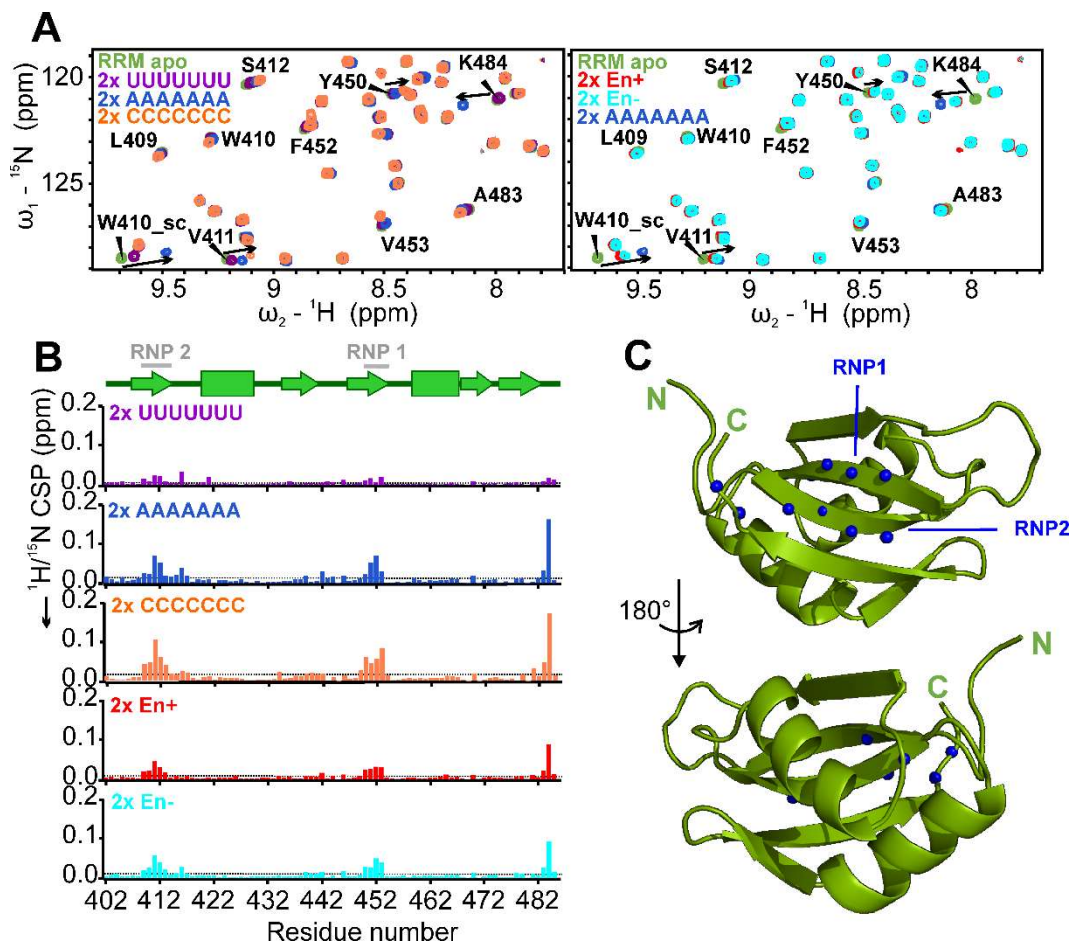


Figure 4. RNA-binding preferences of SAFB2 RRM domain. (A) ^1H - ^{15}N HSQC zoom-ins showing overlays of 2x molar excess of RNAs as indicated (color code depicted in upper left corner) with the apo RRM, respectively. Assignments for RNP residues involved in RNA interaction are included. Addition of G₇ resulted in severe precipitation and was thus not evaluated accordingly. See Supplementary Figure 4 for full spectra. (B) CSP plots of individual RNAs over amino acid sequence. Indicated above is the RRM secondary structure with RNP1 and RNP2 highlighted. Dotted grey lines represent the CSP average. (C) Mapping of the ten significantly perturbed CS for residues shown as spheres (N-atoms) to a structural model of the SAFB2 RRM domain, i.e. the lowest energy representative of a CS-Rosetta-derived ensemble (Supplementary Figure 2). Maximum CSPs locate to the two RNP motifs and the C-terminal tail, respectively.

2.5. Target DNA-preferences of SAP

We next sought to determine the DNA-binding capability of the SAFB2 putative DBD, SAP (Table 1). EMSAs with fluorescently labelled 13-mer DNAs, either GC- or AT-rich, reveal SAP's slight preference for an AT over GC-rich content (Figure 5A). More pronounced, the SAFB2 SAP domain exclusively binds double stranded DNA, clearly distinguishing it from other described SAP domains [38]. Since SAP's apparent micromolar DNA-binding affinity makes it well suitable for NMR-analysis, we next aimed at atom-resolved insights into SAP-DNA interaction. Stepwise titration of AT-rich dsAT_{16mer} up to 6-fold molar excess confirmed SAP binding to occur in a fast-exchange regime – indicative of micromolar affinity – for the majority of peaks (Figure 5B). We then quantified CSPs and mapped significantly affected residues on the lowest-energy CS-Rosetta structure of the SAP domain (Figure 5C and D). As expected, we found only few, but yet-more significantly perturbed residues upon interaction with the AT-rich dsAT_{16mer} DNA. The same residues were perturbed upon interaction with GC-rich dsGC_{16mer}, showing identical trajectories but overall lower maximum CSP values (Supplementary Figure 5). No interaction was observed with ssAT_{16mer}, in line with the suggested preferences from the EMSAs. Furthermore, reducing the DNA length to 9 base pairs did not alter CSP values compared to the 16mer, indicating dsAT_{9mer} to be sufficient for interaction with the SAP interface (Supplementary Figure 5B).

Interestingly, three of the significantly perturbed residues cluster on one side of the domain and include the two highly conserved residues Lys53 and Ser54, plus an additional serine (Ser31) located in the N-terminal helical extension (Figure 1B). A fourth strongly affected residue, Arg45, was found on the opposite side of the domain. This suggests either a complex type of interaction between SAP and DNA, an indirect effect derived from intra-domain rearrangements upon DNA-binding or a significant change in chemical environment based on SAP inter-domain interactions.

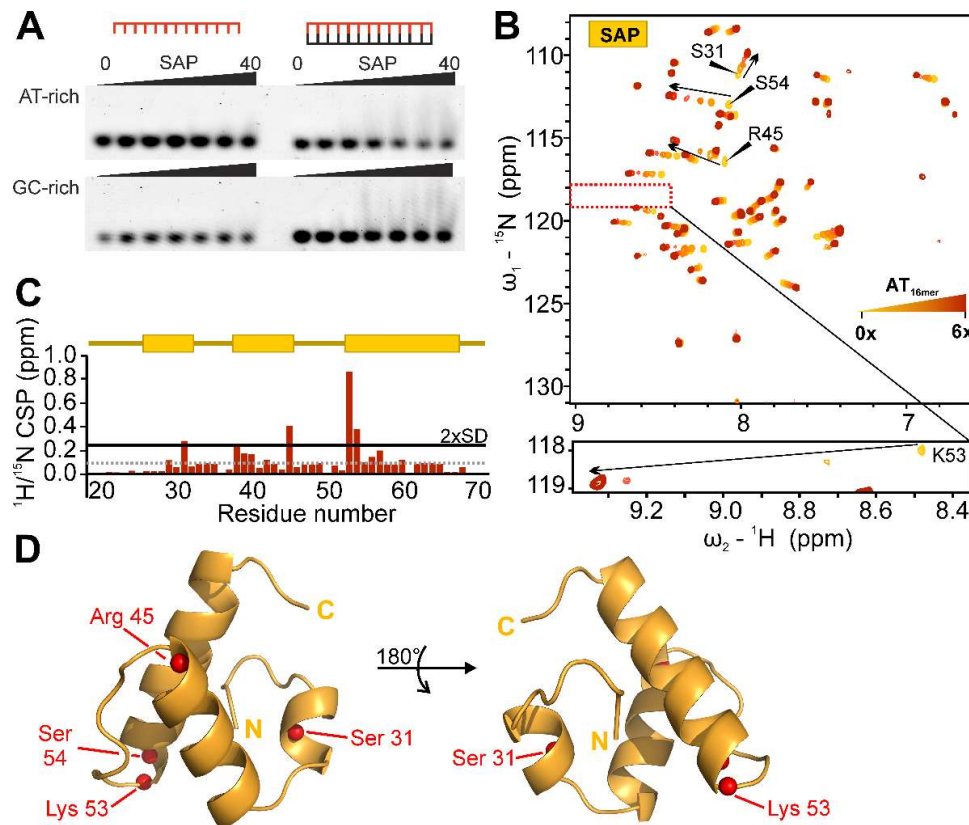


Figure 5. DNA-binding preferences of the SAFB2 SAP domain. (A) EMSAs of SAP domain with ss (left) and ds (right) DNAs, either AT-rich (top) or GC-rich (bottom) (with *sense* sequences as follows: AT_{13mer} 5'-GCAATAAATACG-3'; GC_{13mer} 5'-GCCCCCGCGCCCG-3'). Increasing SAP protein concentration (0-40 μM) is indicated by black bar above gel

pictures. EMSAs shown here are representatives of technical triplicates (Supplementary Fig. 6). **(B)** ^1H - ^{15}N HSQC of SAP titrated with increasing molar ratios (0.5x, 1x, 1.85x, 4x, 6x) of the dsDNA AT_{16mer} (5'-GCGCACAATATAACGC-3'). The most perturbed resonances are labelled with their assignments. The zoom-in of the boxed region below shows the highly conserved Lys 53 (see Figure 1), which is most strongly affected. Note the different contour levels used to visualize this peak relative to the settings of the full spectrum above. **(C)** CSP plots of 6x molar excess of AT_{16mer} plotted over amino acid sequence. Indicated above is the SAP secondary structure. Dotted grey line represents the CSP average, the solid black line the threshold of 2x standard deviation. **(D)** Mapping of the four significantly perturbed CS for residues shown as spheres (N-atoms) plotted on a structural model of the SAFB2 SAP domain. The structural model is the lowest energy representative of a CS-Rosetta-derived ensemble.

2.6. The SAFB2 RRM domain binds both NA types

For both NBDs of SAFB2, we found moderate. i.e. micromolar affinities with the expected nucleic acid type – RNA for the RRM and DNA for the SAP domain. Since many RRM domains are well described to bind also DNA, we next wondered about potential cross-interactions of the SAFB2 RRM and SAP domains with the non-dedicated nucleic acid, respectively.

We first used a bona-fide 34 nt model RNA ('Bulge', Table 1) with a well-distributed sequence composition and comprising linear as well as structured regions [53]. A two-fold excess of Bulge-RNA resulted in significant CSPs of the RRM domain (Supplementary Fig. 5A). In contrast, SAP does not interact with Bulge-RNA, as indicated by the lack of CSPs (Supplementary Fig. 5). Together with the results shown in Figure 5, this specifies SAFB2 SAP as an obligate dsDBD.

Vice versa, we then analyzed the RRM interacting with DNA. To that end, we used single- or double-stranded 16mer DNAs, either AT- or GC-rich (Table 1, dsAT_{16mer}, ssAT_{16mer} and dsGC_{16mer}). Interestingly, the obtained CSP patterns indicate the RRM binds those DNAs with an equal extent and pattern of CSP compared to the tested poly-N RNAs (A₇ and C₇) in chapter 2.4 (Supplementary Fig. 5) pointing at an identical interaction surface. Similarly, the RRM seemingly exhibits an identical binding mode for both NA types, as suggested by the same CSP trajectories. The dual binding potential of SAFB2's RRM domain might pose a particular and unique mechanistic feature.

2.7 SAP domain mutational study

Based on our titration of SAP with the AT_{16mer} dsDNA (Fig. 5) we next examined the contribution of single amino acids within the SAP domain to DNA binding. Therefore, we created mutants based on the CSP plot (Fig. 5C) where we mutated the four amino acids with the highest CSPs to an alanine (S31A, R45A, K53A/S54A). The two neighboring amino acids K53/S54 were combined in one mutant. The influence of these amino acid exchanges on the domain fold was investigated by comparing ^1H - ^{15}N HSQC spectra of the mutant SAP domains with the SAP_{WT} spectrum, respectively (Supplementary Figure 7). Clearly, for SAP_{S31A} and SAP_{K53A/S54A} mutants the folded state of the SAP domain is not impaired. Surprisingly, this was different for SAP_{R45A}, which lost secondary structure based on the confined distribution of peaks in the HSQC, indicating a majorly unfolded species.

We next analyzed the mutants' abilities to bind dsDNA. Therefore, we recorded ^1H - ^{15}N HSQCs at 6- (SAP_{R45A} and SAP_{K53A/S54A}) or 8-fold (SAP_{S31A}) molar excess of AT_{9mer} and compared them to their respective apo spectra (Figure 6). The lack of significant CSPs for SAP_{K53A/S54A} suggests both residues to be decisive for DNA-interaction. This is well in line with literature, proposing the SAP DNA-interface to comprise the loop and the tips of both helices of the helix-loop-helix [38,54,55]. In contrast to that, SAP_{S31A} shows CSPs with trajectories comparable to the WT (Figure 6 and Supplementary Figure 8) but with a lower total extent, which indicates that Ser31 increases SAP's DNA-binding affinity, but is not inevitably crucial for interac-

tion. Lastly, SAP_{R45A} shows minor CSPs, which suggests some interaction of the protein with DNA. However, due to the loss of structure in the mutant the protein-DNA interaction is most likely non-specific and rather based on the re-exposed positive charges of SAP residues engaging with the negatively charged DNA backbone.

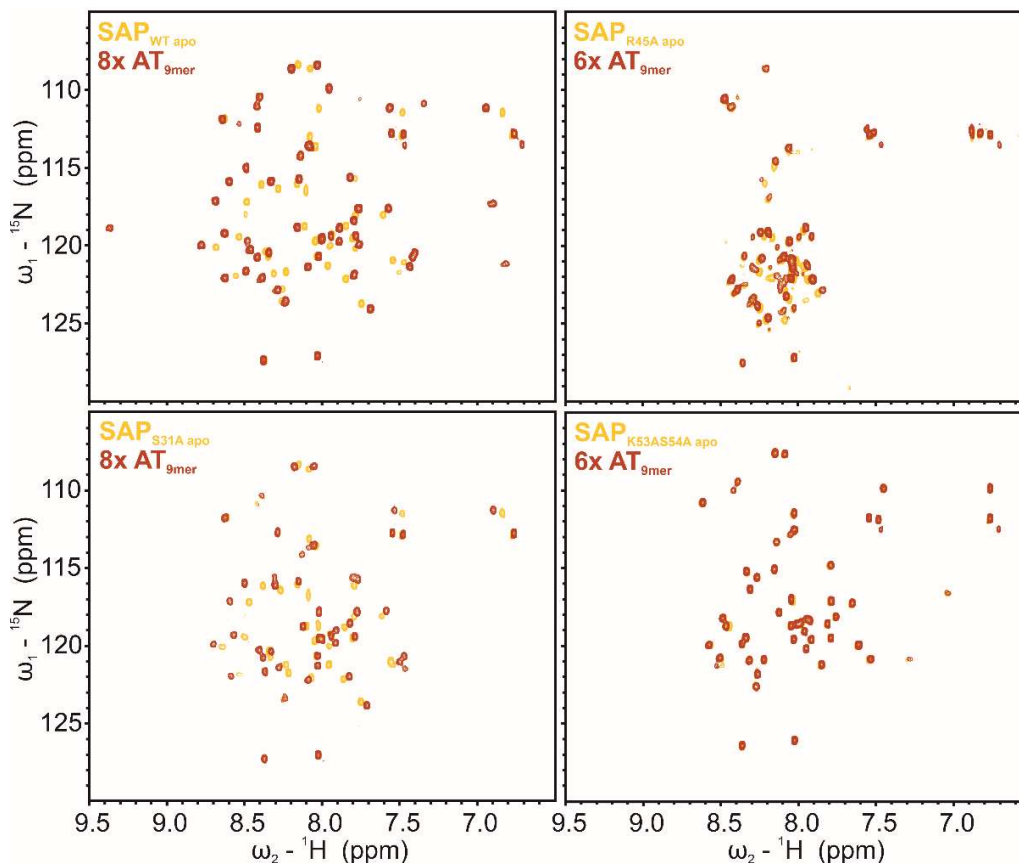


Figure 6. DNA-binding capacity of SAFB2 SAP domain mutants. ^1H - ^{15}N HSQC overlay of apo SAP proteins (yellow) with 6- or 8-fold excess (red) of AT_{9mer} (5'-CAATATAAC-3'), as indicated. Top panel: SAP_{WT} (left) and SAP_{R45A} (right); bottom panel: SAP_{S31A} (left) and SAP_{K53A/S54A} (right). Contour levels are scaled individually between proteins, for peaks to be visible. Contour levels for each apo and DNA-bound spectra of one protein are adjusted.

2.8. SAP domain intra- and inter-domain dynamics

SAFB2 has been shown to form dimers, if not higher oligomers, including heteromultimers with the other SAFB proteins [16,56,57]. While earlier work suggested the prominent CC region to convey protein-protein interactions, there is to date no full confinement of sequence stretches with regard to this. In fact, nucleic acid-binding specificity and affinity might be fostered by the coordinated recognition of adjacent motifs, i.e. dimers/oligomers of folded domains with or without DNA/RNA. RRM domains are known to exist in a broad bandwidth of facets including the capability of mediating protein interactions. We thus first examined if the SAFB2 RRM domain exists in a monomeric form and used NMR ^{15}N relaxation experiments to determine the total correlation time, a strong measure for the underlying molecular weight. We found a homogenous relaxation behavior of folded RRM residues supporting the compact domain. The average correlation time of 5.3 ns is clearly in accordance with a monomeric protein (Supplementary Figure 9).

For the SAP domain, we first performed in-detail inspection of their apparent oligomeric states via elution volumes in analytical size exclusion chromatography (aSEC) (Figure 7A). Surprisingly, molecular weight estimation based on calibration of an aSEC column with standards suggests that both the wild-type

and the mutant SAP versions do not run as monomeric species (Figure 7B and Supplementary Figure 11G), but with MWs between monomers and dimers. This observation could indicate either protein oligomeric mixes or non-canonical retention by the column based on non-globular shapes, e.g. loss of compactness by intra-domain movements between the helices or even partial unfolding. The latter is best seen by the broad elution volume of SAP_{R45A} is in line with the loss of fold observed in the ¹H-¹⁵N HSQC (Supplementary Figure 7) and revealing an apparent MW larger than a dimer in aSEC. Nevertheless, this phenomenon is also seen for the N-terminally truncated SAP (26-70), suggesting that the diminished retention is caused by the core SAP fold, likely the HLH motif itself.

To obtain further insight into SAP oligomeric and dynamic features we recorded ¹⁵N relaxation data for wild-type SAP (21-70). Surprisingly, individual amides show a large spread in R2 values leading to a large standard deviation (>50%). Motions on a μ s-ms timescale can affect chemical exchange and thereby influence R2 rates. We thus measured R1rho relaxation, which allows to partially clear R2 from exchange contributions. Interestingly, we obtained an average total correlation time of 6 ns (Supplementary Figure 10), estimating an apparent MW of 10kDa in contrast to the theoretical 5.7 kDa, which represents a domain dimer, rather than a monomer. However, the individual amides still show a large spread in R1rho rates (Supplementary Figure 10). We thus assume that the monomeric domain undergoes partial dimerization and experiences dynamics in form of exchange phenomena leading to the large variance in R2 rates. Of note, the obtained R1rho data for SAP WT yielded consistently reduced transversal relaxation rates, but a basically identical pattern throughout the domain suggesting that SAP exists in dynamic equilibrium between conformers and /or a monomer-dimer mix.

To finally obtain experimental molecular weights, we subjected all SAP version samples to SEC-MALS. As a result, the identified molar masses were in good agreement with monomers for all versions of SAP (Figure 7C), but all MWs are slightly higher than the theoretical ones (Supplementary Figure 11A-F). We thus conclude that the elution volumes observed in aSEC runs reflect both a mixture of monomer and dimer as well as intra-domain breathing, and SEC-MALS derived MWs are that of monomer with the influence of a low percentage of dimer in exchange. The latter is in line with the suggested intra- and inter-domain exchange observed via NMR relaxation experiments (Supplementary Figure 10).

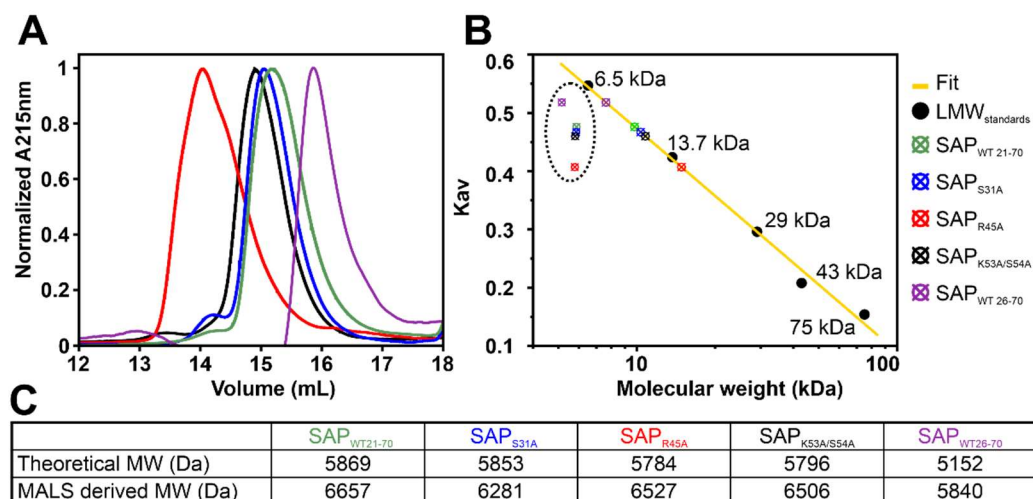


Figure 7. SAFB2 SAP domain mutants. (A) aSEC of SAP wild-type domain and mutants. **(B)** Estimation of molecular weights (MW) based on MW standards. Shown is the partition coefficient (K_{av}) plotted as a function of MW. Colored points (in dotted circle) are the K_{av} from aSEC over their theoretical MWs (expecting a monomer), respectively. Colored points on the fit line are the MWs determined based on the calibration standards (see also Supplementary Figure 11). **(C)** SEC-MALS derived MWs of the SAP wild-type domain and mutants compared to their theoretical MWs of a monomer.

3. Discussion

SAFB proteins are known for more than three decades and found involved in a large variety of nucleic acid-related biological functions [16], specifically in the context of stress response. Their supposedly dual nucleic acid-binding potential has made them bona fide anchoring proteins to locate and regulate DNA- and RNA-processing events, best represented by SAFB2 [23,58]. How exactly this is organized through SAFBs as integrators of RNA-, DNA- and protein-binding has majorly remained elusive, while atom-resolved pictures would yield valuable insights into the respective functions. Surprisingly, for none of the three members experimental structural information had been publicly available.

In this work we provide the first atom-resolved analysis of the SAFB2 SAP and RRM domains and their dedicated interactions with DNA and RNA, respectively, as summarized graphically in Figure 8. Enabled by the suitable size of the two domains, we used NMR to unravel residue-resolved information of nucleic-acid binding and provide chemical shift-derived structural models. NMR is ideally applicable in this early step of a systematic deciphering of structure-guided contributions of the multi-domain SAFB2 to its integrated functions [59,60]. It was here used to optimize domain boundaries, determine the fold and oligomer states of domains, map their nucleic acid binding sites and report on internal dynamic regions.

For the SAFB2 RRM domain we were able to obtain a complete protein backbone NMR assignment at pH 6.5. The few missing residues at pH 5.0 (see BMRB entry 51701) cluster in an exposed region of the compact domain, and likely line-broadening under the acidic experimental conditions is caused by exchanging conformations. Interestingly, we find lysine 470, located centrally in this cluster, strongly affected by RNA-binding. This suggests the local flexibility to be a determinant for the effective engagement with target sequences, and the entropic barrier of conformational selection acts as a proofread for specificity. The SAFB2 RRM, based on its preliminary sparse data-derived structure, appears to comprise a canonical fold and well compares to the majority of RRM structures. Thus, our identification of the binding interface with the tested RNAs collectively suggests an interaction via the RNP motifs, supported by possible contacts via flanking residues as e.g. K470. Yet, we cannot rule out additional flanking regions or extensions [61] to modulate fold and RNA motif recognition [32] as also shown very recently [62]. Similarly, e.g. SR proteins pseudo-RRMs were shown to use non-canonical RNA-binding sites [63].

The herein suggested motif preference of the SAFB2 RRM is found in C-rich or A-rich RNA sequences. As such it is possibly reflected by the ENCODE-deposited C-rich 5-/6-mers derived from RBnS, and also found for RRM structures earlier [51], e.g. for the IMP3 MD-RBP [52,64]. However, we find weaker interaction with the precise ENCODE RNA, and likewise a control RNA with a central motif, e.g. recognized by the SRSF6 RRM structures [65], is bound to an equal extent. This may hint at a hidden parameter for specificity in a protein fl-context (see below). Alternatively, we suggest that poly-C or poly-A allow for an optimal stacking of ssRNA to be accommodated on the RRM surface and interaction could involve additional contacts. Thus, the central motif covered by the RNPs may be found more affine as represented by the RBnS-derived fl-directed motif.

Current literature still leaves much space for the actual role of RNA-binding by the SAFB2 RRM in a functional context. Hutter et al. find that the RRM is not needed in pre-miRNA processing by SAFB2 [23] indicating the interaction with RNA takes place via the IDRs or mediated via interacting proteins. Still, a direct RNA-interacting role could become more evident in other SAFB2 contexts or within protein multimers, as shown for the CAC-recognition by RRM structures in dimeric RBPMS [51]. Of note however, our NMR relaxation data clearly reveal the SAFB2 RRM to exist as a monomer in its isolated form. Alternatively, the SAFB RRM may be targeting RNAs in a structured context. In fact, the possibility of RBDs, specific for ssRNA, to interact with ssRNA embedded in structures has been shown for RRM structures before [66] and is also

discussed for KH domains [67,68]. Of a special note, our data suggest the RRM can in principle interact with DNA. Although highly speculative, it could thus in principle fulfill moonlighting roles as an additional (facultative) DBD or even switch between the two types of nucleic acids in dependence of the cellular context and relevant mechanistic requirements. A similar assumption has earlier been made for KH domains, most recently within the helicase DDX43 [69].

We succeeded in the full backbone NMR resonance assignment of the SAFB2 SAP domain. Based on that we were able to construct a high-confidence CS-Rosetta structural ensemble, that suggests an extended SAP domain of an otherwise canonical fold, conserved fold, and this is also corroborated by the unstructured N-terminal part of our starting construct (AA 21-25). We however find an interesting CSP pattern w.r.p. to prominent contacts in the N-terminal helical extension, as supported by our mutational data. Extended SAP domains with N-terminal helical extensions have been found earlier (see e.g. our DALI-derived examples used for alignments), and even appear to comprise a full third helix in their amino-terminus [50]. However, involvement of this extension in DNA-binding has remained unresolved or not even discussed for the majority of them. The crystal structure of the T4 endonuclease in complex with a Holliday junction dsDNA shows the analogous N-terminal extension of its SAP domain facing the DNA interface [70], and this domain is a close structural relative of the SAFB2 SAP. Despite a large number of available high-resolution structures of SAP domains, both isolated and in larger multi-domain contexts deposited in the PDB [71], complexes with DNA have remained the exceptions or do not reveal the SAP domain itself in direct contact with DNA. Interestingly, a very recent study shows that C-terminal basic residues of the SDE2 SAP contribute to DNA-recognition [38]. Our NMR data unambiguously prove the SAFB2 SAP domain to specifically interact with dsDNA, while ssDNA is not recognized despite a very similar fold compared to the solution structure of SDE2 SAP (Supplementary Fig. 2H) [38].

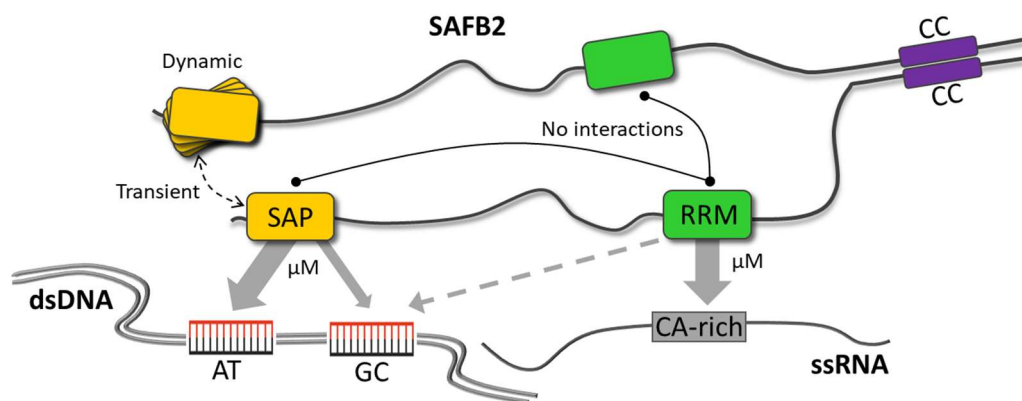


Figure 8: Summary of nucleic acid-binding preferences of SAFB2 SAP and RRM domains as determined in this study. In addition, possible inter- and intramolecular domain interactions based on current experimental knowledge are depicted. SAFB2 and its domains are not shown in scale.

Interestingly, the SAFB2 SAP domain (21-70) only shares 36/57% of sequence identity/similarity with the SAP domain of SAFA. Nonetheless, the available NMR structure (PDB 1ZRJ, unpublished) well represents the overall fold of the sparse data-derived model of SAFB2 SAP in this work (r.m.s.d. 1.1 Å between Val35 and Val64) and moreover contains a likewise oriented N-terminal helical extension. This not only suggests a highly conserved fold of extended SAP types, but also a possible evolutionary relation between the two SAF subfamilies. Of note however, the SAFA SAP does not contain a serine at its analogous position 31, indicating a role of this residue for DNA sequence specificity.

The most surprising finding here is the existence of the SAFB2 SAP domain intra- and inter-domain dynamics revealed by NMR. To our knowledge, this has not specifically been addressed for available SAP domain structures. We observe chemical exchange, possibly caused by movements of the two HL helices with respect to each other, that may keep the domain flexible towards the recognition of correct DNA sequences. The domain could thus adopt to non-canonical DNA structure or adjust to modified groove widths. Inter-helical contacts may thus be transient or facultative, while a set of defined interactions are needed for structural integrity. As such, the crucial residue Arg45 may be involved in salt bridges, which will explain SAP domain unfolding after mutation.

Likewise, transient conditional SAP dimers will have interesting functional consequences for SAFB2. First, a second dimerization site apart from the previously described CC [16,23] raises the question of the physiologically present oligomer state(s) of SAFB2 (Figure 8). In fact, multiple dimerization sites would facilitate meshes, which could serve an architectural basis in SAFB2-contained granule formation, and provide a complex surface pattern for specifically 3D-arranged RNAs and DNAs. Second, dimeric SAP allows for a significantly higher specificity for interaction with dsDNA stretches. The latter could be represented by a tandem motif, a palindromic sequence or looped DNA. Also, full SAP domain dimerization may be initiated by a cognate tandem DNA motif. Certainly, SAFB2 oligomers will be able to engage with nucleic acids at higher affinities (avidity-driven) than the low-to-medium μM -range we find for individual domains and their target motifs in this study. Full experimental structures of SAP with DNA will reveal functionally relevant pictures of SAFB2 at the chromatin.

Additional protein-protein interactions of functional impact could exist between the SAFB2 SAP and RRM domains. Indeed, RRMs have been shown to mediate protein-protein interactions earlier [72]. Clearly, we do not see any evidence for interaction based on NMR data for the exact SAFB2 domain constructs under study (Supplementary Figure 12). However, we do not rule out interactions between sequence stretches more distal, respectively, which may bring the two domains in close contact in a natural context.

The latter would yet more put into focus the question of SAFB2 simultaneously interacting with DNA and RNA. In general, such interchanging or mixed D/RNPs including SAFBs could couple the events of specific local chromatin remodeling, transcriptional regulation of miR genes and the subsequent processing of nascent transcripts, or as shown very recently in an example for the interplay of nuclear matrix remodeling and mRNA splicing regulation [58] by SAFB2. In this regard, a close proximity of both bindings might be favored and give rise to a functional switch, in that internal protein interactions are crucial for the formation of correct D/RNPs.

SAFBs may represent yet another example of integrative protein functions as also known for other (e.g. immune-modulatory) DRBPs that act on multiple levels of gene regulation (summarized in [2]). SAFBs, though, may have very distinct functions, and some of those will relate to its capability of simultaneous NA-binding in respective cellular contexts. Performing NA-binding tasks might also be steered by protein co-factors that direct SAFBs to the correct sites of action in cells. Similarly, lncRNAs could act in an architectural manner to locate SAFBs to DNA-related tasks. In fact, lncRNA-binding has been shown for SAFBs in the most recent past [20,73].

Interestingly, binding of SAFBs has very recently been brought in correlation with the nuclear localization of linear, mostly unspliced RNAs [74] indicating an anchoring role for them in transcript processing, which is susceptible to stress in a reversible manner [58].

Based on our initial structural data, we envision future work with distinct derivable questions towards the role of SAP and RRM and their oligomeric states will stepwise unravel the SAFB2-driven cellular mechanisms in more detail and at the atomic level.

4. Materials and Methods

4.1. Construct design

Amino acid sequences of the human SAFB2 SAP and RRM domains were taken from UniProt [35] entry Q14151. SAP domain boundaries were initially designed to comprise aa 21 to 70, in analogy to human MKL/myocardin-like protein 1 SAP domain (PDB 2KVU, unpublished). RRM domain boundaries were designed to comprise aa 404 to 485, in analogy to the human RBM5 RRM domain (PDB 2LKZ, [43]). For both domains, *E. coli* codon-optimized DNA constructs (Eurofins Genomics) were cloned into pET21-based vector pET-Trx1a, containing an N-terminal His₆-tag, a Thioredoxin-tag (Trx) and a TEV cleavage site *via* restriction digestion (*NcoI/XhoI*, New England BioLabs®) and subsequent ligation (T4 DNA Ligase, New England BioLabs®). For SAP, a 5.87 kDa protein was obtained theoretically upon TEV-cleavage, containing three artificial N-terminal residues (GAM), before the start of the native protein sequence at G21 of full-length SAFB2. For RRM, a 9.13 kDa protein was obtained upon TEV-cleavage, containing three artificial N-terminal residues (GAM) before the start of the native protein sequence at G404 of full-length SAFB2.

SAP domain point mutations (S31A, R45A and K53A/S54A) were either cloned by site-directed mutagenesis (SDM) SPRINP [75] or according to NEBaseChanger® SDM following the manufacturer's protocol. The oligonucleotides used for SDM are listed in SI Table 1. Like the WT SAP protein, the mutant sequences were preceded by the three artificial residues GAM.

An N-terminally truncated SAP domain (26-70) was cloned based on pET-Trx1a SAP 21-70 plasmid using oligonucleotides listed in SI Table 1, according to NEBaseChanger® following the manufacturer's protocol. For SAP₂₆₋₇₀, a 5.15 kDa protein was theoretically obtained upon TEV-cleavage, with a direct start of the native protein sequence at G26 of full-length SAFB2.

4.2. Protein production

Uniformly (¹³C),¹⁵N-labelled SAP and RRM were expressed in *E. coli* strain BL21 (DE3) in M9 minimal medium containing (2 g/L ¹³C₆-D-glucose (Eurisotop)), 1 g/L ¹⁵NH₄Cl (Cambridge Isotope Laboratories) and 50 µg/mL kanamycin. Protein expression was induced with 1mM IPTG at OD₆₀₀ 0.6 to 0.8 for 18 to 21 h at 18°C. Cell pellets were resuspended in 50 mM Tris, pH 8, 300 mM NaCl and 10mM imidazole, supplemented with 100 µL protease inhibitor mix (SERVA) and 100 µg DNase per 1 L culture. RRM buffer was additionally supplemented with 3mM β-mercaptoethanol. Cells were disrupted by sonication. Supernatants were cleared by centrifugation (40 min, 9000 × g, 4 °C). The cleared supernatants were passed over a Ni²⁺-NTA gravity flow column (Sigma-Aldrich) and the His₆-Trx-tag was cleaved over night at 4 °C with 0.5 mg of TEV protease per 1 L of culture, while dialyzing into fresh buffer 50 mM Tris, pH 8, 300 mM NaCl (and 3mM β-mercaptoethanol for RRM). With a second Ni²⁺-NTA gravity flow column, TEV protease and the cleaved tag were removed. The SAFB2 SAP and RRM domains were further purified via size exclusion on a HiLoad 16/600 SD 75 (GE Healthcare), respectively. SAP size exclusion was carried out in 25 mM sodium phosphate, 150 mM NaCl, 0.02% NaN₃, pH 6.5. Pure SAP containing fractions were determined by SDS-PAGE. According to its retention volume in the size exclusion chromatography, the 5.87 kDa-protein is a dimer in solution. Based on calibration, the peak position of SAP corresponded to an approximate size of 11 kDa, which is in good agreement with the theoretical molecular mass of the dimeric protein (11.74 kDa). The SEC fractions of interest were pooled and concentrated using Amicon® centrifugal concentrators (molecular weight cutoff 3 kDa). NMR samples were prepared in 25 mM sodium phosphate, 150 mM NaCl, 0.02% NaN₃, pH 6.5 with 5% (v/v) D₂O.

RRM size exclusion was carried out in 20 mM Bis-Tris, 1 M NaCl, 2 mM TCEP, 0.02% NaN₃, pH 6.5. Pure RRM containing fractions were determined by SDS-PAGE and accordingly pooled and concentrated

using Amicon® centrifugal concentrators (molecular weight cutoff 3 kDa). NMR samples were prepared in 20 mM Bis-Tris, 150 mM NaCl, 2 mM TCEP, 0.02% NaN₃, pH 6.5 with 5% (v/v) D₂O.

4.3. RNA preparation

RNA 6-mers and 7-mers (Table 1) were obtained from Dharmacon Horizon in 2 μmol quantities, deprotected and desalted. According to the yields, each RNA was dissolved in MQ-H₂O to reach a final concentration of 2mM. RNA was transferred to Pur-A-Lyzer Midi 1000 tube (Sigma Aldrich) and dialyzed twice against a volume of 1L H₂O. Concentration was determined with a NanoDrop (Thermo Fischer) subsequently to NMR titration experiments.

The Bulge-RNA was produced by in-house optimized *in vitro* transcription and purified as described previously [53,76]. Final RNA samples were buffer exchanged to either SAP or RRM NMR buffer conditions and homogeneity and stability monitored by denaturing PAGE.

4.4. NMR data acquisition and analysis

All software applications related to structural biology, including structure visualization and image creation by PyMol (Schrödinger) have been run via the SGrid platform [77]. NMR measurements were carried out at the Frankfurt BMRZ using Bruker spectrometers of 600-950 MHz proton Larmor frequency, equipped with cryogenic probes and using Z-axis pulsed field gradients. All SAFB2 SAP protein samples were consistently measured in buffer of 20 mM BisTris, 150 mM NaCl, pH 6.5 including 5% of D₂O at 298K. For the SAP backbone assignment (BMRB entry: 51700), we recorded the following experiments: HNcaCO, HNCO, HNCACB, HNcoCACB, HBHANH, HBHAcoNH and ¹⁵N-NOESY. The RRM was measured in the identical buffer, but including 2 mM of TCEP. The initial backbone assignment was performed at pH 5.0 (BMRB entry: 51701) using an HNcaCO, HNCO, HNCACB, and a ¹⁵N-NOESY experiments and amide assignments transferred to pH 6.5 via pH titration and an additional ¹⁵N-NOESY experiment (BMRB entry: 51724). Data acquisition and processing was undertaken using Topspin versions 3 and 4. Cosine-squared window functions were applied for apodization in all dimensions. Spectra were referenced with respect to added DSS and for ¹³C/¹⁵N as suggested in [78].

¹H-¹⁵N HSQC experiments for the SAP domain were acquired with a nitrogen offset at 118 ppm and a constant spectral width of 26 ppm using 96-128 indirect complex points. For the RRM domain, we used a nitrogen offset at 116 ppm and a constant spectral width of 30 ppm using 112-128 indirect complex points.

For nucleic acid titrations to the SAFB2 SAP domain, we used 96 indirect points and complex forward linear prediction until 144 points. For SAFB2 RRM titrations, 112 indirect points were recorded with complex forward linear prediction until 168 points. A reference HSQC was recorded prior to measuring the final titration point. Subsequently, apo and end-point samples were re-mixed to obtain distinct stoichiometric ratios. Combined ¹H/¹⁵N chemical shift perturbations were calculated using the formula:

$$CSP = \sqrt{\left(\frac{\delta N}{5}\right)^2 + (\delta H)^2}$$

The {¹H}¹⁵N heteronuclear NOE experiments were performed as interleaved HSQC-based pseudo-3D versions including solvent suppression by WATERGATE sequence [79] and a saturation delay of 6 s from samples of 1mM (SAP) and 600 μM (RRM) concentration. Analysis of CSPs and hetNOE ratios were performed using the CCPNMR analysis 2.4 software suite [80] and the program Sparky [81].

For ^{15}N -relaxation data of the SAFB2 SAP domain, we used the following T1 delays: 20, 40, 80, 100, 200, 400, 600, 1000, and 1400 ms. T2 delays were 16.96, 33.92, 67.84, 101.76, 135.68, 169.6, 203.52, 271.36, and 339.2 ms. T1rho delays were 4, 8, 12, 24, 48, 72, 96, 120, 144 ms. All data were acquired with implemented temperature compensation at a field strength of 700 MHz proton Larmor frequency and 298K. Relaxation Rates R1, R2 and R1rho were derived as reciprocals of either T1, T2 and T1rho. For the SAFB2 RRM domain, relaxation data were recorded at 600 MHz proton Larmor frequency and 298K. The following delays were used for T1: 10, 50, 150, 250, 350, 500, 750, 1500, and 2500 ms and for T2: 33.92, 50.88, 67.84, 84.8, 101.76, 118.72, 135.68, 152.64, 169.6, 203.52, and 271.36 ms. TauC values were calculated according to the formula:

$$\text{TauC (ns)} = \left(\frac{1}{2 * \pi * 15N * 10^9} \right) \sqrt{\left(\frac{3}{2} \right) * \left(\frac{R2}{R1} \right) - \left(\frac{7}{6} \right) * 10^9}$$

*15N referring to the ^{15}N Larmor frequency.

CS-based structural models were calculated using the CS-Rosetta [82] web server version 3.8 (version 3.3 of the CS-Rosetta toolbox) by generating 3000 structures and no automated trimming of flexible tails. No additional restraints other than chemical shifts as deposited for the two domains and TALOS-derived backbone angles have been added. For both domains the auto-validation returned a converged run, indicating a meaningful structural ensemble. Statistics of both runs' ensembles of best 10 structures are shown in Supplementary Figure 2. The full output is publicly available (see Data availability statement).

4.5. Analytical Size-exclusion chromatography (aSEC) and aSEC-based MW determination

A Superdex75 Increase 10/300GL column was calibrated at 4°C by loading 100µL of premixed commercial low molecular weight standards (Cytiva) containing a mixture of five proteins in SAP buffer and running them at 0.6 mL/min using a Bio-Rad NGC FPLC. A standard calibration curve was generated from a linear fit of the partition coefficient (Kav) versus the log molecular weight of the protein standards. The apparent molecular weight of SAP wild-type and the mutants was calculated by interpolation from their Kav using the equation derived from the linear fit. Analytical SEC runs at 4°C were performed by loading 100 µL of protein samples (~4 mg/mL) onto a Superdex75 Increase 10/300GL equilibrated with SAP buffer using a Bio-Rad NGC FPLC at 0.6 mL/min. The run was monitored with UV absorbance at 215nm. All aSEC runs were analyzed using ChromLab_v6 (Bio-Rad) and the traces plotted in OriginPro (see Supplementary Fig. 12).

4.6. Size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS)

SEC-MALS was performed at 4 °C using a Superdex75 Increase 10/300GL column, a light-scattering detector (TREOS) and refractometer (Optilab rEX) from Wyatt Technology and a UV detector, HPLC pump and degasser from Jasco. System was equilibrated with 3 column volumes of SAP buffer. All buffers were filtered through 0.1 µm pore size Durapore PVDF membrane filters (Merck) following a recirculation through the system for 20 h at 0.5 mL/min to improve the baseline by removing air bubbles and particles by degasser and pre-injection filter (0.1 µm). Per measurement 270 µg of protein in 100 µL buffer were injected and analyzed at a flow rate of 0.5 mL/min. Light scattering detector was calibrated by using monomeric BSA (Merck). The obtained signals were processed with the ASTRA software package version 5.3.4.13 (Wyatt Technology). The concentration was determined by the refractometer using a refractive index increment dn/dc at 20.5 °C and 658 nm as calculated by SEDFIT v16.1c [83].

4.7. Electromobility shifts assays (EMSAs)

EMSAs for the SAP-domain were performed with DNA such that the single strand or one of the two strands in dsDNA was 5'-labeled with fluorescein (6-FAM, Sigma-Aldrich) (Table 1). For each reaction, 200 pmol of either single- or double-stranded DNA were mixed with 0,6 µg of yeast tRNA (Roche), 10 mM MgCl₂ and SAP-buffer (25 mM sodium phosphate, 150 mM NaCl, 0.02% NaN₃, pH 6.5)/protein in different volumes/concentrations to match a final reaction volume of 20 µl. Additionally, 3 µl of loading buffer were added immediately prior to loading the samples (10 µl of each reaction) onto a 6% polyacrylamide gel. Gel-electrophoresis was run for 50 min at 80 V. Finally, the gels were imaged inside the glass plates with a Typhoon Imager (GE Healthcare) with a laser at 488 nm excitation and an emission filter at 520 nm.

Supplementary Materials: Supporting information can be downloaded together with this manuscript.

Author Contributions: Conceptualization, S.M.K. and A.S.; methodology, S.M.K., J.v.E., K.D., J.N.T., R.A., A.S.; validation, S.M.K., J.v.E., K.D., R.A., A.S.; formal analysis, A.S.; software, A.S.; data curation, A.S.; writing—original draft preparation, S.M.K., J.v.E, K.D. and A.S.; writing—review and editing, S.M.K and A.S.; visualization, S.M.K., J.v.E., K.D., J.N.T., A.S.; supervision, A.S.; project administration, S.M.K. and A.S.; funding acquisition, A.S. All authors have read and agreed to the published version of the manuscript.

Funding: The Frankfurt BMRZ acknowledges support by the state of Hesse. This work was supported by the German Research Council (DFG) through DFG grant number SCHL2062/2-1 to A.S., the Goethe University Excellence Initiative “SCALE”, and by the Johanna Quandt Young Academy at Goethe through financial support of A.S. (stipend number 2019/AS01).

Institutional Review Board Statement: Ethical review and approval are not applicable for this study.

Informed Consent Statement: Not applicable.

Data Availability Statement: Full results of CS-Rosetta runs for SAP and RRM domains are accessible under entries <https://csrosetta.bmr.io/entry/36b19517843f> and <https://csrosetta.bmr.io/entry/54b3a1e3a6cb>, respectively. Backbone chemical shifts of SAFB2 SAP and RRM have been deposited in the BMRB with IDs 51700, 51701 and 51724, respectively.

Acknowledgments: We thank Katharina Targaczewski for excellent technical wet lab support.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Xiao, R.; Chen, J.Y.; Liang, Z.; Luo, D.; Chen, G.; Lu, Z.J.; Chen, Y.; Zhou, B.; Li, H.; Du, X.; et al. Pervasive Chromatin-RNA Binding Protein Interactions Enable RNA-Based Regulation of Transcription. *Cell* **2019**, *178*, 107-121 e118, doi:10.1016/j.cell.2019.06.001.
2. Hudson, W.H.; Ortlund, E.A. The structure, function and evolution of proteins that bind DNA and RNA. *Nat Rev Mol Cell Biol* **2014**, *15*, 749-760, doi:10.1038/nrm3884.

3. Davis, B.N.; Hilyard, A.C.; Nguyen, P.H.; Lagna, G.; Hata, A. SMAD proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. *Mol. Cell* **2010**, *39*.
4. Shi, L.; Godfrey, W.R.; Lin, J.; Zhao, G.; Kao, P.N. NF90 regulates inducible IL-2 gene expression in T cells. *J. Exp. Med.* **2007**, *204*.
5. Shim, J.; Lim, H.; Yates Iii, J.; Karin, M. Nuclear export of NF90 is required for interleukin-2 mRNA stabilization. *Mol. Cell* **2002**, *10*.
6. Kuo, P.H.; Doudeva, L.G.; Wang, Y.T.; Shen, C.K.J.; Yuan, H.S. Structural insights into TDP43 in nucleic-acid binding and domain interactions. *Nucleic Acids Res.* **2009**, *37*.
7. Lukavsky, P.J. Molecular basis of UG-rich RNA recognition by the human splicing factor TDP43. *Nature Struct. Mol. Biol.* **2013**, *20*.
8. Huang, D.B. Crystal structure of NF- κ B (p50)₂ complexed to a high-affinity RNA aptamer. *Proc. Natl Acad. Sci. USA* **2003**, *100*.
9. Müller, C.W.; Rey, F.A.; Sodeoka, M.; Verdine, G.L.; Harrison, S.C. Structure of the NF- κ B p50 homodimer bound to DNA. *Nature* **1995**, *373*.
10. Kiledjian, M.; Dreyfuss, G. Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box. *EMBO J* **1992**, *11*, 2655-2664.
11. Romig, H.; Fackelmayer, F.O.; Renz, A.; Ramsperger, U.; Richter, A. Characterization of SAF-A, a novel nuclear DNA binding protein from HeLa cells with high affinity for nuclear matrix/scaffold attachment DNA elements. *EMBO J* **1992**, *11*, 3431-3440, doi:10.1002/j.1460-2075.1992.tb05422.x.
12. Renz, A.; Fackelmayer, F.O. Purification and molecular cloning of the scaffold attachment factor B (SAF-B), a novel human nuclear protein that specifically binds to S/MAR-DNA. *Nucleic Acids Res* **1996**, *24*, 843-849, doi:10.1093/nar/24.5.843.
13. Weighardt, F.; Cobianchi, F.; Cartegni, L.; Chiodi, I.; Villa, A.; Riva, S.; Biamonti, G. A novel hnRNP protein (HAP/SAF-B) enters a subset of hnRNP complexes and relocates in nuclear granules in response to heat shock. *J Cell Sci* **1999**, *112 (Pt 10)*, 1465-1476, doi:10.1242/jcs.112.10.1465.
14. Watanabe, K.; Ohtsuki, T. Inhibition of HSF1 and SAFB Granule Formation Enhances Apoptosis Induced by Heat Stress. *Int J Mol Sci* **2021**, *22*, doi:10.3390/ijms22094982.
15. Drakouli, S.; Lyberopoulou, A.; Papatheanassiou, M.; Mylonis, I.; Georgatsou, E. Enhancer of rudimentary homologue interacts with scaffold attachment factor B at the nuclear matrix to regulate SR protein phosphorylation. *FEBS J* **2017**, *284*, 2482-2500, doi:10.1111/febs.14141.
16. Norman, M.; Rivers, C.; Lee, Y.B.; Idris, J.; Uney, J. The increasing diversity of functions attributed to the SAFB family of RNA-/DNA-binding proteins. *Biochem J* **2016**, *473*, 4271-4288, doi:10.1042/BCJ20160649.
17. Huo, X.; Ji, L.; Zhang, Y.; Lv, P.; Cao, X.; Wang, Q.; Yan, Z.; Dong, S.; Du, D.; Zhang, F.; et al. The Nuclear Matrix Protein SAFB Cooperates with Major Satellite RNAs to Stabilize Heterochromatin Architecture Partially through Phase Separation. *Mol Cell* **2020**, *77*, 368-383 e367, doi:10.1016/j.molcel.2019.10.001.
18. Hernandez-Hernandez, J.M.; Mallappa, C.; Nasipak, B.T.; Oesterreich, S.; Imbalzano, A.N. The Scaffold attachment factor b1 (Safb1) regulates myogenic differentiation by facilitating the transition of myogenic gene chromatin from a repressed to an activated state. *Nucleic Acids Res* **2013**, *41*, 5704-5716, doi:10.1093/nar/gkt285.
19. Hong, E.A.; Gautrey, H.L.; Elliott, D.J.; Tyson-Capper, A.J. SAFB1- and SAFB2-mediated transcriptional repression: relevance to cancer. *Biochem Soc Trans* **2012**, *40*, 826-830, doi:10.1042/BST20120030.
20. Luo, S.; Zhang, M.; Wu, H.; Ding, X.; Li, D.; Dong, X.; Hu, X.; Su, S.; Shang, W.; Wu, J.; et al. SAIL: a new conserved anti-fibrotic lncRNA in the heart. *Basic Res Cardiol* **2021**, *116*, 15, doi:10.1007/s00395-021-00854-y.

21. Zhou, M.; Kuruvilla, L.; Shi, X.; Viviano, S.; Ahearn, I.M.; Amendola, C.R.; Su, W.; Badri, S.; Mahaffey, J.; Fehrenbacher, N.; et al. Scaffold association factor B (SAFB) is required for expression of prenyltransferases and RAS membrane association. *Proc Natl Acad Sci U S A* **2020**, *117*, 31914-31922, doi:10.1073/pnas.2005712117.
22. Yang, J.S.; Qian, C.; You, S.; Rotinen, M.; Posadas, E.M.; Freedland, S.J.; Di Vizio, D.; Kim, J.; Freeman, M.R. Scaffold attachment factor B1 regulates androgen degradation pathways in prostate cancer. *Am J Clin Exp Urol* **2021**, *9*, 337-349.
23. Hutter, K.; Lohmuller, M.; Jukic, A.; Eichin, F.; Avci, S.; Labi, V.; Szabo, T.G.; Hoser, S.M.; Huttenhofer, A.; Villunger, A.; et al. SAFB2 Enables the Processing of Suboptimal Stem-Loop Structures in Clustered Primary miRNA Transcripts. *Mol Cell* **2020**, *78*, 876-889 e876, doi:10.1016/j.molcel.2020.05.011.
24. Xiong, F.; Wang, R.; Lee, J.H.; Li, S.; Chen, S.F.; Liao, Z.; Hasani, L.A.; Nguyen, P.T.; Zhu, X.; Krakowiak, J.; et al. RNA m(6)A modification orchestrates a LINE-1-host interaction that facilitates retrotransposition and contributes to long gene vulnerability. *Cell Res* **2021**, *31*, 861-885, doi:10.1038/s41422-021-00515-8.
25. Oesterreich, S. Scaffold attachment factors SAFB1 and SAFB2: Innocent bystanders or critical players in breast tumorigenesis? *J Cell Biochem* **2003**, *90*, 653-661, doi:10.1002/jcb.10685.
26. Hashimoto, T.; Matsuda, K.; Kawata, M. Scaffold attachment factor B (SAFB)1 and SAFB2 cooperatively inhibit the intranuclear mobility and function of ERalpha. *J Cell Biochem* **2012**, *113*, 3039-3050, doi:10.1002/jcb.24182.
27. Peidis, P.; Voukkalis, N.; Aggelidou, E.; Georgatsou, E.; Hadzopoulou-Cladaras, M.; Scott, R.E.; Nikolakaki, E.; Giannakouros, T. SAFB1 interacts with and suppresses the transcriptional activity of p53. *FEBS Lett* **2011**, *585*, 78-84, doi:10.1016/j.febslet.2010.11.054.
28. Fang, W.; Bartel, D.P. MicroRNA Clustering Assists Processing of Suboptimal MicroRNA Hairpins through the Action of the ERH Protein. *Mol Cell* **2020**, *78*, 289-302 e286, doi:10.1016/j.molcel.2020.01.026.
29. Erhardt, S.; Stoeklin, G. The heat's on: nuclear stress bodies signal intron retention. *EMBO J* **2020**, *39*, e104154, doi:10.15252/emboj.2019104154.
30. Burge, C.B. RNA Bind-n-Seq (RBNS) pulldown experiment against SAFB2. *The ENCODE project* **2020**, *ENCSR558RBK*, doi:10.17989/ENCSR558RBK.
31. Lambert, N.; Robertson, A.; Jangi, M.; McGeary, S.; Sharp, P.A.; Burge, C.B. RNA Bind-n-Seq: quantitative assessment of the sequence and structural binding specificity of RNA binding proteins. *Mol Cell* **2014**, *54*, 887-900, doi:10.1016/j.molcel.2014.04.016.
32. Clery, A.; Blatter, M.; Allain, F.H. RNA recognition motifs: boring? Not quite. *Curr Opin Struct Biol* **2008**, *18*, 290-298, doi:10.1016/j.sbi.2008.04.002.
33. Maris, C.; Dominguez, C.; Allain, F.H.T. The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. *FEBS J.* **2005**, *272*.
34. Fu, X.D. SAFB ChIP-seq in K562. *The ENCODE project* **2017**, *ENCSR072VUO*, doi:doi:10.17989/ENCSR072VUO.
35. UniProt, C. UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Res* **2021**, *49*, D480-D489, doi:10.1093/nar/gkaa1100.
36. Sievers, F.; Wilm, A.; Dineen, D.; Gibson, T.J.; Karplus, K.; Li, W.; Lopez, R.; McWilliam, H.; Remmert, M.; Soding, J.; et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* **2011**, *7*, 539, doi:10.1038/msb.2011.75.
37. Aravind, L.; Koonin, E.V. SAP - a putative DNA-binding motif involved in chromosomal organization. *Trends Biochem Sci* **2000**, *25*, 112-114, doi:10.1016/s0968-0004(99)01537-6.
38. Weinheimer, A.S.; Paung, Y.; Rageul, J.; Khan, A.; Lo, N.; Ho, B.; Tong, M.; Alphonse, S.; Seeliger, M.A.; Kim, H. Extended DNA-binding interfaces beyond the canonical SAP domain contribute to the function of

- replication stress regulator SDE2 at DNA replication forks. *J Biol Chem* **2022**, *298*, 102268, doi:10.1016/j.jbc.2022.102268.
39. Wishart, D.S.; Bigam, C.G.; Yao, J.; Abildgaard, F.; Dyson, H.J.; Oldfield, E.; Markley, J.L.; Sykes, B.D. 1H, 13C and 15N chemical shift referencing in biomolecular NMR. *J Biomol NMR* **1995**, *6*, 135-140, doi:10.1007/BF00211777.
40. Korn, S.M.; Dhamotharan, K.; Furtig, B.; Hengesbach, M.; Lohr, F.; Qureshi, N.S.; Richter, C.; Saxena, K.; Schwalbe, H.; Tants, J.N.; et al. (1)H, (13)C, and (15)N backbone chemical shift assignments of the nucleic acid-binding domain of SARS-CoV-2 non-structural protein 3e. *Biomol NMR Assign* **2020**, *14*, 329-333, doi:10.1007/s12104-020-09971-6.
41. Korn, S.M.; Lambertz, R.; Furtig, B.; Hengesbach, M.; Lohr, F.; Richter, C.; Schwalbe, H.; Weigand, J.E.; Wohnert, J.; Schlundt, A. (1)H, (13)C, and (15)N backbone chemical shift assignments of the C-terminal dimerization domain of SARS-CoV-2 nucleocapsid protein. *Biomol NMR Assign* **2021**, *15*, 129-135, doi:10.1007/s12104-020-09995-y.
42. Metzler, W.J.; Constantine, K.L.; Friedrichs, M.S.; Bell, A.J.; Ernst, E.G.; Lavoie, T.B.; Mueller, L. Characterization of the three-dimensional solution structure of human profilin: 1H, 13C, and 15N NMR assignments and global folding pattern. *Biochemistry* **1993**, *32*, 13818-13829, doi:10.1021/bi00213a010.
43. Song, Z.; Wu, P.; Ji, P.; Zhang, J.; Gong, Q.; Wu, J.; Shi, Y. Solution structure of the second RRM domain of RBM5 and its unusual binding characters for different RNA targets. *Biochemistry* **2012**, *51*, 6667-6678, doi:10.1021/bi300539t.
44. Nerli, S.; McShan, A.C.; Sgourakis, N.G. Chemical shift-based methods in NMR structure determination. *Prog Nucl Magn Reson Spectrosc* **2018**, *106-107*, 1-25, doi:10.1016/j.pnmrs.2018.03.002.
45. Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Zidek, A.; Potapenko, A.; et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021**, *596*, 583-589, doi:10.1038/s41586-021-03819-2.
46. Lange, O.F.; Rossi, P.; Sgourakis, N.G.; Song, Y.; Lee, H.W.; Aramini, J.M.; Ertekin, A.; Xiao, R.; Acton, T.B.; Montelione, G.T.; et al. Determination of solution structures of proteins up to 40 kDa using CS-Rosetta with sparse NMR data from deuterated samples. *Proc Natl Acad Sci U S A* **2012**, *109*, 10873-10878, doi:10.1073/pnas.1203013109.
47. Holm, L.; Rosenstrom, P. Dali server: conservation mapping in 3D. *Nucleic Acids Res* **2010**, *38*, W545-549, doi:10.1093/nar/gkq366.
48. Ge, H.; Zhou, D.; Tong, S.; Gao, Y.; Teng, M.; Niu, L. Crystal structure and possible dimerization of the single RRM of human PABPN1. *Proteins* **2008**, *71*, 1539-1545, doi:10.1002/prot.21973.
49. Thomsen, N.D.; Lawson, M.R.; Witkowsky, L.B.; Qu, S.; Berger, J.M. Molecular mechanisms of substrate-controlled ring dynamics and substepping in a nucleic acid-dependent hexameric motor. *Proc Natl Acad Sci U S A* **2016**, *113*, E7691-E7700, doi:10.1073/pnas.1616745113.
50. Raaijmakers, H.; Toro, I.; Birkenbihl, R.; Kemper, B.; Suck, D. Conformational flexibility in T4 endonuclease VII revealed by crystallography: implications for substrate binding and cleavage. *J Mol Biol* **2001**, *308*, 311-323, doi:10.1006/jmbi.2001.4592.
51. Teplova, M.; Farazi, T.A.; Tuschl, T.; Patel, D.J. Structural basis underlying CAC RNA recognition by the RRM domain of dimeric RNA-binding protein RBPMS. *Q Rev Biophys* **2016**, *49*, e1, doi:10.1017/S0033583515000207.
52. Schneider, T.; Hung, L.H.; Aziz, M.; Wilmen, A.; Thaum, S.; Wagner, J.; Janowski, R.; Muller, S.; Schreiner, S.; Friedhoff, P.; et al. Combinatorial recognition of clustered RNA elements by the multidomain RNA-binding protein IMP3. *Nat Commun* **2019**, *10*, 2266, doi:10.1038/s41467-019-09769-8.

53. Tants, J.N.; Becker, L.M.; McNicoll, F.; Muller-McNicoll, M.; Schlundt, A. NMR-derived secondary structure of the full-length Ox40 mRNA 3'UTR and its multivalent binding to the immunoregulatory RBP Roquin. *Nucleic Acids Res* **2022**, *50*, 4083-4099, doi:10.1093/nar/gkac212.
54. Notenboom, V.; Hibbert, R.G.; van Rossum-Fikkert, S.E.; Olsen, J.V.; Mann, M.; Sixma, T.K. Functional characterization of Rad18 domains for Rad6, ubiquitin, DNA binding and PCNA modification. *Nucleic Acids Res* **2007**, *35*, 5819-5830, doi:10.1093/nar/gkm615.
55. Zhang, Z.; Zhu, L.; Lin, D.; Chen, F.; Chen, D.J.; Chen, Y. The three-dimensional structure of the C-terminal DNA-binding domain of human Ku70. *J Biol Chem* **2001**, *276*, 38231-38236, doi:10.1074/jbc.M105238200.
56. Townson, S.M.; Dobrzycka, K.M.; Lee, A.V.; Air, M.; Deng, W.; Kang, K.; Jiang, S.; Kioka, N.; Michaelis, K.; Oesterreich, S. SAFB2, a new scaffold attachment factor homolog and estrogen receptor corepressor. *J Biol Chem* **2003**, *278*, 20059-20068, doi:10.1074/jbc.M212988200.
57. Sergeant, K.A.; Bourgeois, C.F.; Dalglish, C.; Venables, J.P.; Stevenin, J.; Elliott, D.J. Alternative RNA splicing complexes containing the scaffold attachment factor SAFB2. *J Cell Sci* **2007**, *120*, 309-319, doi:10.1242/jcs.03344.
58. Taze, C.; Drakouli, S.; Samiotaki, M.; Panayotou, G.; Simos, G.; Georgatsou, E.; Mylonis, I. Short-term hypoxia triggers ROS and SAFB mediated nuclear matrix and mRNA splicing remodeling. *Redox Biol* **2022**, *58*, 102545, doi:10.1016/j.redox.2022.102545.
59. Daubner, G.M.; Clery, A.; Allain, F.H. RRM-RNA recognition: NMR or crystallography...and new findings. *Curr Opin Struct Biol* **2013**, *23*, 100-108, doi:10.1016/j.sbi.2012.11.006.
60. Gobl, C.; Madl, T.; Simon, B.; Sattler, M. NMR approaches for structural analysis of multidomain proteins and complexes in solution. *Prog Nucl Magn Reson Spectrosc* **2014**, *80*, 26-63, doi:10.1016/j.pnmrs.2014.05.003.
61. Martinez-Lumbreras, S.; Taverniti, V.; Zorrilla, S.; Seraphin, B.; Perez-Canadillas, J.M. Gbp2 interacts with THO/TREX through a novel type of RRM domain. *Nucleic Acids Res* **2016**, *44*, 437-448, doi:10.1093/nar/gkv1303.
62. Duszczyc, M.M.; Wischnewski, H.; Kazeeva, T.; Arora, R.; Loughlin, F.E.; von Schroetter, C.; Pradere, U.; Hall, J.; Ciaudo, C.; Allain, F.H. The solution structure of Dead End bound to AU-rich RNA reveals an unusual mode of tandem RRM-RNA recognition required for mRNA regulation. *Nat Commun* **2022**, *13*, 5892, doi:10.1038/s41467-022-33552-x.
63. Clery, A.; Sinha, R.; Anczukow, O.; Corrionero, A.; Moursy, A.; Daubner, G.M.; Valcarcel, J.; Krainer, A.R.; Allain, F.H. Isolated pseudo-RNA-recognition motifs of SR proteins can regulate splicing using a noncanonical mode of RNA recognition. *Proc Natl Acad Sci U S A* **2013**, *110*, E2802-2811, doi:10.1073/pnas.1303445110.
64. Jia, M.; Gut, H.; Chao, J.A. Structural basis of IMP3 RRM12 recognition of RNA. *RNA* **2018**, *24*, 1659-1666, doi:10.1261/ma.065649.118.
65. Alvelos, M.I.; Bruggemann, M.; Sutandy, F.R.; Juan-Mateu, J.; Colli, M.L.; Busch, A.; Lopes, M.; Castela, A.; Aartsma-Rus, A.; Konig, J.; et al. The RNA-binding profile of the splicing factor SRSF6 in immortalized human pancreatic beta-cells. *Life Sci Alliance* **2021**, *4*, doi:10.26508/lsa.202000825.
66. Allain, F.H.; Gubser, C.C.; Howe, P.W.; Nagai, K.; Neuhaus, D.; Varani, G. Specificity of ribonucleoprotein interaction determined by RNA folding during complex formulation. *Nature* **1996**, *380*, 646-650, doi:10.1038/380646a0.
67. Teplova, M.; Malinina, L.; Darnell, J.C.; Song, J.; Lu, M.; Abagyan, R.; Musunuru, K.; Teplov, A.; Burley, S.K.; Darnell, R.B.; et al. Protein-RNA and protein-protein recognition by dual KH1/2 domains of the neuronal splicing factor Nova-1. *Structure* **2011**, *19*, 930-944, doi:10.1016/j.str.2011.05.002.
68. Korn, S.M.; Ulshofer, C.J.; Schneider, T.; Schlundt, A. Structures and target RNA preferences of the RNA-binding protein family of IGF2BPs: An overview. *Structure* **2021**, *29*, 787-803, doi:10.1016/j.str.2021.05.001.

69. Yadav, M.; Singh, R.S.; Hogan, D.; Vidhyasagar, V.; Yang, S.; Chung, I.Y.W.; Kusalik, A.; Dmitriev, O.Y.; Cygler, M.; Wu, Y. The KH domain facilitates the substrate specificity and unwinding processivity of DDX43 helicase. *J Biol Chem* **2021**, *296*, 100085, doi:10.1074/jbc.RA120.015824.
70. Biertumpfel, C.; Yang, W.; Suck, D. Crystal structure of T4 endonuclease VII resolving a Holliday junction. *Nature* **2007**, *449*, 616-620, doi:10.1038/nature06152.
71. Berman, H.M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T.N.; Weissig, H.; Shindyalov, I.N.; Bourne, P.E. The Protein Data Bank. *Nucleic Acids Res* **2000**, *28*, 235-242, doi:10.1093/nar/28.1.235.
72. Muto, Y.; Yokoyama, S. Structural insight into RNA recognition motifs: versatile molecular Lego building blocks for biological systems. *Wiley Interdiscip Rev RNA* **2012**, *3*, 229-246, doi:10.1002/wrna.1107.
73. Huang, Y.; Qiao, Y.; Zhao, Y.; Li, Y.; Yuan, J.; Zhou, J.; Sun, H.; Wang, H. Large scale RNA-binding proteins/LncRNAs interaction analysis to uncover lncRNA nuclear localization mechanisms. *Brief Bioinform* **2021**, *22*, doi:10.1093/bib/bbab195.
74. Ron, M.; Ulitsky, I. Context-specific effects of sequence elements on subcellular localization of linear and circular RNAs. *Nat Commun* **2022**, *13*, 2481, doi:10.1038/s41467-022-30183-0.
75. Edelheit, O.; Hanukoglu, A.; Hanukoglu, I. Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. *BMC Biotechnol* **2009**, *9*, 61, doi:10.1186/1472-6750-9-61.
76. Wacker, A.; Weigand, J.E.; Akabayov, S.R.; Altincekic, N.; Bains, J.K.; Banijamali, E.; Binas, O.; Castillo-Martinez, J.; Cetiner, E.; Ceylan, B.; et al. Secondary structure determination of conserved SARS-CoV-2 RNA elements by NMR spectroscopy. *Nucleic Acids Res* **2020**, *48*, 12415-12435, doi:10.1093/nar/gkaa1013.
77. Morin, A.; Eisenbraun, B.; Key, J.; Sanschagrin, P.C.; Timony, M.A.; Ottaviano, M.; Sliz, P. Collaboration gets the most out of software. *Elife* **2013**, *2*, e01456, doi:10.7554/eLife.01456.
78. Wishart, D.S.; Sykes, B.D. Chemical-Shifts as a Tool for Structure Determination. In *Nuclear Magnetic Resonance, Pt C; Methods in Enzymology*; 1994; Volume 239, pp. 363-392.
79. Sklenar, V.; Bax, A. Spin-echo water suppression for the generation of pure-phase two-dimensional NMR spectra. *Journal of Magnetic Resonance* **1987**, *74*, 469-479, doi:10.1016/0022-2364(87)90269-1.
80. Vranken, W.F.; Boucher, W.; Stevens, T.J.; Fogh, R.H.; Pajon, A.; Llinas, P.; Ulrich, E.L.; Markley, J.L.; Ionides, J.; Laue, E.D. The CCPN data model for NMR spectroscopy: Development of a software pipeline. *Proteins-Structure Function and Bioinformatics* **2005**, *59*, 687-696.
81. Lee, W.; Tonelli, M.; Markley, J.L. NMRFAM-SPARKY: enhanced software for biomolecular NMR spectroscopy. *Bioinformatics* **2015**, *31*, 1325-1327, doi:10.1093/bioinformatics/btu830.
82. Shen, Y.; Lange, O.; Delaglio, F.; Rossi, P.; Aramini, J.M.; Liu, G.; Eletsky, A.; Wu, Y.; Singarapu, K.K.; Lemak, A.; et al. Consistent blind protein structure generation from NMR chemical shift data. *Proc Natl Acad Sci U S A* **2008**, *105*, 4685-4690, doi:10.1073/pnas.0800256105.
83. Zhao, H.; Casillas, E., Jr.; Shroff, H.; Patterson, G.H.; Schuck, P. Tools for the quantitative analysis of sedimentation boundaries detected by fluorescence optical analytical ultracentrifugation. *PLoS One* **2013**, *8*, e77245, doi:10.1371/journal.pone.0077245.