Nucleotide-amino acid π-stacking interactions initiate photo cross-linking in RNA-protein complexes

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Photo-induced cross-linking is a mainstay technique to characterize RNA-protein interactions. However, UV-induced cross-linking between RNA and proteins at “zero-distance” is poorly understood. Here, we investigate cross-linking of the RBFOX alternative splicing factor with its hepta-ribonucleotide binding element as a model system. We examine the influence of nucleobase, nucleotide position and amino acid composition using CLIR-MS technology (crosslinking-of-isotope-labelled-RNA-and-tandem-mass-spectrometry), that locates cross-links on RNA and protein with site-specific resolution. Surprisingly, cross-linking occurs only at nucleotides that are π-stacked to phenylalanines. Notably, this π-stacking interaction is also necessary for the amino-acids flanking phenylalanines to partake in UV-cross-linking. We confirmed these observations in several published datasets where cross-linking sites could be mapped to a high resolution structure. We hypothesize that π-stacking to aromatic amino acids activates cross-linking in RNA-protein complexes, whereafter nucleotide and peptide radicals recombine. These findings will facilitate interpretation of cross-linking data from structural studies and from genome-wide datasets generated using CLIP (cross-linking-and-immunoprecipitation) methods.
The human genome encodes more than 1500 RNA binding proteins (RBPs) that regulate key processes, including translation, localisation, stability and splicing. In order to understand fully the structure-function relationship of an RBP, it is necessary to identify to which RNA it binds in vivo, and how non-covalent interactions occur in the binding site. RNA–protein binding occurs at conserved RNA binding domains, such as RNA recognition motifs (RRM), heterogeneous nuclear ribonucleoprotein (hnRNP) K-homology domains and zinc finger (ZnF) domains. These domains recognize short, usually single-stranded regions of 3–8 nucleotides (nt) known collectively as consensus RNA binding elements (RBEs) that often contain degenerate positions. Additional binding affinity and selectivity can be generated via supplementary contacts between the RNA and the protein; for example, the RBP FUS has a bipartite binding mode comprising its ZnF domain and its RRM. RNA–protein binding has also been observed with proteins that lack canonical RNA binding domains (RBDs). Taken together, these features render difficult the prediction of an RBP’s substrates based only on a computational search for its consensus RBE. Indeed, recent studies of the RBFOX protein family showed that only one-half of the isolated RNA targets contain the RBFOX consensus binding motif and that other motifs presumably are responsible for some of its splicing activities.

Many state-of-the-art methods to identify RNA-protein interactions in vivo employ RNA-protein cross-linking induced by UV light. For example, by combining UV cross-linking with mass spectrometry approaches, proteins bound to given RNAs can be identified. Conversely, UV cross-linking and immunoprecipitation (CLIP) protocols are commonly used to identify RNA-binding sites for given proteins on a transcriptome-wide scale. Technical advances constantly improve these techniques, however, a long-standing challenge in structure/mechanism-oriented studies is to identify the points of cross-linking on both the RNA and the protein with site-specific resolution. Recently, we (RA, AL, FA) introduced cross-linking of segmentally isotope-labelled RNA and tandem mass spectrometry (CLIR-MS), which identifies the sites of amino acid/ribonucleotide cross-links in a single protocol.

The photo-induced reaction between amino acids and ribonucleotides occurs between free radical species at “zero distance”. Reactions involve mainly uridines and guanosines, but most amino acids can participate. Nevertheless, cross-links typically only occur at specific positions in the RNA-RBP motif, for which there is currently no mechanistic rationale. Moreover, it has proven difficult to investigate and identify factors that promote cross-linking, largely because i) the RNA-protein binding site environment, which is critical for cross-linking chemistry, cannot be simulated in simple solvents, and ii) the chemistry usually produces complex product mixtures that are difficult to characterize on a background of protein and nucleic acid UV damage.

Here, we investigate the structural requirements for the cross-linking of an RNA to its RBP partner. We use the RRM domain of the RBFOX family (FOXRRM) and its RNA consensus binding motif U$_9$G$_3$C$_3$A$_3$U$_6$G$_6$U$_7$ (FOXRRM) as a model system, exploiting the high affinity of the complex forms and its well-characterized NMR structure. We introduce $^{13}$C-labelled ribonucleotides into the FOXRRM heptanucleotide and use CLIR-MS to identify RNA-protein cross-links with site-specific resolution. Cross-linking on the protein clusters at amino acids around two phenylalanines, consistent with previous findings. However, with few exceptions, it only occurs on the RNA at U$_9$, G$_3$ and G$_6$. We then employ site-specific mutagenesis to probe systematically the influence of nucleobase, nucleotide position and amino acid composition on the cross-linking profile. This reveals that cross-linking only occurs with guanosine or uridine at three of the seven nucleotide positions, and only when bases are stacked to amino acid side chains. Remarkably, this primary stacking interaction is required for neighbouring amino acids to participate in cross-linking. We identify and confirm the importance of this structural feature in selected published examples from other groups as well as an unbiased analysis of three large datasets, suggesting that it is of primary importance for zero-length cross-linking in native RNA–protein binding sites. Moreover, we expect that this finding will facilitate the interpretation of RNA-protein cross-linking data, especially for non-canonical binding motifs. It will also help guide the design of future cross-linking experiments and will aid the development of new tools for de novo motif discovery (see ref. 35).

**Results**

**Optimization of CLIR-MS to identify RNA-protein cross-links with site-specific resolution.** The original CLIR-MS protocol (Fig. 1a) employs RNAs with contiguous regions of differentially isotope-labelled nucleotides in the cross-linking step. After partial RNA and protein digestion, short peptide-oligonucleotide conjugates are identified as matched signal pairs in the precursor ion mass spectrum, which localizes the cross-linked nucleotide to the labelled RNA segment. Overlapping partial sequences then facilitates the localization of the cross-link on the RNA. A drawback of the original implementation of CLIR-MS is the inherent requirement for enzymatic $^{13}$C/$^{15}$N-labelled RNA synthesis (i.e. in vitro transcription). This does not allow site-specific nucleotide labelling which is needed to unambiguously assign the reactive nucleotide. A second limitation is the nuclelease digestion step, which typically produces short oligonucleotides (i.e., 1–4 nt) and is probably less efficient on nucleotides that are structurally changed by cross-linking. In this study, we implemented chemical solutions to help circumvent these problems; we employed $^{13}$C-labelled phosphoramidites (Fig. S1a) during solid-phase RNA synthesis to incorporate labelled nucleotides site-specifically; and we switched from RNase digestion to alkaline hydrolysis of RNA, while exercising care not to degrade the protein or the nucleobases. Consequently, the mass analysis of the product mixtures yielded a greater fraction of peptide-mononucleotide adducts, allowing us to better identify nucleotides that are cross-linked (Fig. S1b).

U$_9$, G$_3$ and G$_6$ in FOXRRM cross-link to amino acids centred on phenylalanines in the FOXRRM. We employed a systematic approach in an effort to identify key structural requirements for cross-linking of RBFOX to RNA. We first used $^{13}$C-labelled versions of FOXRRM to determine all points of reaction between the RNA and the protein. We then synthesized mutated variants of FOXRRM to determine how cross-linking varies with respect to i) the nucleobase, ii) its positions in the RBE, and iii) how it is affected by amino acid composition in the binding site. We were mindful of the fact that mutating sites in the RNA and the protein might alter the mode of (or even abolish) RNA-protein binding, and therefore for each mutant we measured the binding affinity to FOXRRM using surface plasmon resonance spectroscopy (SPR).

We synthesized the seven $^{13}$C-labelled versions of FOXRRM and confirmed the correct incorporation of the label by liquid-chromatography mass spectrometry (LC-MS) (Fig. S2). We incubated each version of FOXRRM with FOXRRM and performed the CLIR-MS protocol. The mass analysis identified short oligonucleotide fragments cross-linked to peptides in clusters close to F126 and F160 (Fig. 1b). Each oligonucleotide signal in the spectrum of Fig. 1b was detected because it contains a $^{13}$C labelled ribose. However, other than for mononucleotides, the
actual site of cross-linking in the fragment could not be called; for example, the tetranucleotide fragment containing A, C, G and U might have cross-linked at any of the four bases (A, G, C or U). We noted that all (>99%) of the detected fragments contained at least one uridine or guanosine, consistent with literature reports\textsuperscript{15,18,32,33,39} that uracil and guanine mainly participate in cross-linking. Here, the use of alkaline hydrolysis for RNA digestion proved advantageous, since it digests a larger fraction of the RNA to mononucleotides (see Fig. S1b, c), thereby identifying unambiguously the cross-linking sites. Hence, focusing only on the mononucleotide species in the spectra of Fig. 1b, revealed that cross-linking in the FOXRBE involved almost uniquely U\textsubscript{1}, G\textsubscript{2} and...
The cross-linking of G₂ and G₆ was consistent with published CLIP data³⁴,⁴⁰ (Fig. 1c). G₂ and G₆ are critical to the binding of FOXRBE to FOXRRM, and their exchange for A₂ or A₆ respectively, greatly reduced protein-binding and cross-linking between the amino acid clusters 126 and 160 and the mutated sites (Fig. S3). Although cross-linking from U₁ was detected in the CLIR-MS experiments, it was hardly observed at U₅ (vide infra) or U₇, consistent with our hypothesis that strict structural parameters govern the photo-induced reactions between FOXRRM and FOXRBE. Isolated cross-links were also observed in some of the spectra of Fig. 1b, c. Although low numbers of cross-links must be considered with caution, their locations suggested that in several cases they were not artifacts. In particular, the cross-links at F₁₁₀ seen with *UGCAUGU and U*GCAUGU (Fig. 1b, top two panels) are consistent with transient (low affinity) binding of U₇G₁₂ in the binding pocket occupied mainly by U₃G₆. Likewise, cross-links around F₁₁₂ in the lower panels of Fig. 1b, c may derive from similarly transient contacts with U₃G₆U₇. One cross-link from P₁₂₅ to cytidine is visible in Fig. 1c. A₄ did not cross-link to FOXRRM (Fig. 1c). Sites of cross-linking at the protein were centered at two phenylalanines (F₁₁₂ and F₁₆₀), with a distribution of 1-3 amino acids flanking these positions³⁸,³⁹. This was confirmed from analysis of the MS/MS spectra in which fragment ions localize the RNA adducts unambiguously on the peptide backbone (Fig. S4).

The cross-links of U₁, G₂ and G₆ aligned well with the NMR structure of FOXRBE bound to FOXRRM³⁶ (Fig. 1d) (PDB ID: 2ERR). The largest number of spectra corresponded to U₁ reacting with P₁₂₅ and F₁₁₂, and to a lesser extent with I₁²₄ and R₁₂₇ (Fig. 1c). Similarly, G₂ cross-linked to I₁²₄, P₁₂₅, F₁₁₂ and R₁₂₇. Of note, U₁ and G₂ each stack to one face of F₁₁₂. Hydrogen bonds are also present between the bases of U₁ and G₂, and between R₁₂₇ and I₁²₄, respectively. G₆ reacted with F₁₁₀ (to which it also stacks), as well as with neighbouring amino acids at positions 158-164; F₁₁₈ contacts the ribose of G₆. Notably, several close RNA-protein contacts that are visible in the NMR structure (i.e. G₆ interacting with F₁₁₂ but not stacking), G₆ stacking with R₁₉₄ and U₅ stacking to H₁₂₀³⁶, did not produce extensive cross-linking.

The current understanding of RNA-protein cross-linking is that close contact between nucleotides and amino acids is the main pre-requisite for a cross-linking event⁴¹,⁴². However, only three from the seven nucleotides of FOXRBE engaged in efficient cross-linking, despite close contacts between all nucleotides and amino acids in the binding site. Hence, we investigated two obvious parameters that could influence cross-linking: i) the chemical reactivities of the nucleobases and the amino acids, and ii) the relative positioning of the reactive pair. By mutating selected nucleotides and amino acids in the binding pocket, we created a cross-linking structure-activity relationship for the FOXRRM-FOXRBE interaction.

Only uridine cross-links to FOXRBE from position 1 of FOXRRM. We synthesized the three labelled mutants of *NGCAUGU (N = A, G, C; Supporting Table 1), as well as the corresponding per-labelled control sequences \( \text{N}^*\text{NG}^*\text{G}^*\text{C}^*\text{A}^*\text{U}^*\text{G}^*\text{U} \). We first confirmed that the NGCAUGU variants bound to FOXRRM using SPR. In this assay, parent UGCAUGU bound strongly to FOXRRM with a \( K_d = 4.1 \text{nM} \). Substitution of the 5'-uridine reduced the strength of the interaction by 4-6-fold for the three variants (AGCAUGU; \( K_d = 24.9 \text{nM} \); CGCAUGU; \( K_d = 22.5 \text{nM} \); GCGCAUGU; \( K_d = 21.3 \text{nM} \)) (Fig. 2a). This was consistent with the NMR structure showing that the 5'-uridine of FOXRBE contributes to binding by \( \pi \)-stacking to F₁₁₂ (Fig. 1d).

Next, we incubated the RNAs together with FOXRRM and irradiated the complexes with increasing doses. Work-up and analysis by SDS-PAGE for the three NGCAUGU mutants revealed a new slow-migrating band on the gels, similar to that of the wild-type FOXRBE (N = U), consistent with RNA-protein cross-linking (Fig. 2b). The appearance of a band on an SDS-PAGE confirms that cross-linking occurs, but it does not identify the site of cross-linking nor the composition of the product. In order to determine whether the mutants cross-linked at the N₁ and N₂ position, we turned to CLIR-MS (Figs. S5, and S6). CLIR-MS data for per-labelled \( \text{N}^*\text{NG}^*\text{G}^*\text{C}^*\text{A}^*\text{U}^*\text{G}^*\text{U} \) confirmed that the three FOXRBE mutants exhibit the same cross-linking “fingerprint” as wild type FOXRBE, i.e. in the same two amino acid clusters around positions 126 and 160 (Fig. S6a). However, in order to differentiate cross-linking of N₁ to that from G₂ in the 126-cluster, we performed CLIR-MS on the singly labelled sequences (*NGCAUGU). In contrast to U₁, cross-linking hardly occurred at A₁, G₁ or C₁ (*UGCA, *GAUGU, *AGCAUGU, resp.), and *G for C₁, A₁, U₁ and U₇ (*UGCA*GAU, *UGCA*G*U, *UGCA*G*U). In each case, we first confirmed that the mutants bound and cross-linked to FOXRRM using SPR and SDS-PAGE gels (Fig. 2d, e, resp., Fig. S6a). Remarkably, in none of these six examples, did the mutated nucleotides cross-link efficiently to the protein (Fig. 2d). The lack of reactivity at U₁ (in *UG*UAUGU) was particularly surprising given the high reactivity of uridine in photo-reactions¹⁸,³². Nevertheless, it was surprising that G₁ was unreactive given the reactivity of G₂, which may have been due to inappropriate orbital overlap in stacking.

In order to determine systematically the propensity for cross-linking at each position in FOXRBE when a photoactive nucleotide (i.e. U or G) is present, we performed CLIR-MS on six additional positional FOXRBE mutants. Thus, we exchanged *U for G₂ and A₄ in FOXRBE (UG*UAUGU, UG*UGU, resp.), and *G for C₁, A₁, U₁ and U₇ (UG*GAU, UG*G*U, UG*G*U, UG*G*U*G, resp.). In each case, we first confirmed that the mutants bound and cross-linked to FOXRRM using SPR and SDS-PAGE gels (Fig. 2d, e, resp., Fig. S6a). Remarkably, in none of these six examples, did the mutated nucleotides cross-link efficiently to the protein (Fig. 2d). The lack of reactivity at U₁ (in *UG*UAUGU) was particularly surprising given the close proximity of C₁ to F₁₁₂ in the NMR structure.

In summary, while G₂ and G₆ in wild type FOXRBE cross-linked to FOXRRM, guanosine did not cross-link efficiently at any other of the other five locations in the FOXRBE. Similarly, uridine readily cross-linked to FOXRRM from position N₁ - where A₁ and G were unreactive - but not from the four other locations in the FOXRBE. Taken together, the data from this controlled model study confirmed that RNA-protein cross-linking events have strict requirements, beyond simply the proximity of a reactive nucleotide and a reactive amino acid.
Aromatic amino acids play a key role in priming RNA-protein cross-linking reactions. Analysis of the aforementioned CLIR-MS data (Figs.1c, and S6) provided two important insights: i) on the RNA side, strong cross-linking only occurred with nucleotides that were stacked to aromatic amino acids; and ii) on the protein side, cross-links involved F126 and F160, but also up to three amino-acids up- and downstream of F126 and F160.

We therefore mutated F126 in FOXRRM to histidine, tyrosine and leucine. An effort to perform CLIR-MS on a tryptophan mutant failed because of protein precipitation. We have previously shown using SPR that aromatic amino acids at position 126 are crucial for binding FOX RBE (F126Y: $K_d = 2.21$ nM; F126H: $K_d = 25.9$ nM), although a sterically-fitting aliphatic amino acid such as leucine can partially substitute for the phenylalanine (F126L: $K_d = 374$ nM)36. We irradiated these variants in the presence of FOXRBE. All three protein mutants cross-linked to FOXRBE, as evident from SDS-PAGE (Fig. 3a).

Next, we carried out CLIR-MS experiments with uniformly $^{13}$C-labelled FOXRBE. F126Y and F126H cross-linked to the FOXRBE similarly to FOXRRM (Fig. 3b). The cross-linking profile was similar for the three complexes at F126 and F160. However, when phenylalanine was exchanged for leucine, binding was weaker and the cross-linking to position 126 was abolished. Notably, cross-linking to the neighbouring amino acids at positions 124, 125 and 127.

Interestingly, the F126H mutant appears not to cross-link to G2, as shown by the absence of G mononucleotides (brown) or CG dinucleotides (turquoise) in Fig.3b (Supporting Data 1). Although we do not have supporting data, nor know of any precedence in literature, it is plausible that the histidine has a different cross-linking preference to those of tyrosine or phenylalanine and/or that stacking to the guanosine G2 is disturbed in this particular complex. Unexpectedly, a H120 cross-link occurred with the three FOXRRM mutants, which was hardly observable in the wild type FOXRBM (Fig. 3b). Analysis of the oligonucleotide fragments in Fig. 3b strongly suggested that the cross-link occurred with U5. In fact, the NMR structure of FOXRBM-FOXRB shows that U5 adopts a stacking arrangement with H120, and thus might have

Fig. 2 FOXRRM/FOXRBE cross-linking is nucleotide- and site-specific. a SPR sensorgrams of FOXRBE variants bound to FOXRRM. b SDS-PAGE gels showing that all N1-variants undergo cross-linking with FOXRRM with increasing radiation. The cross-linking product band is indicated by “XL” (repeated three times). c CLIR-MS plots show that cross-linking occurs at U1 of FOXRBE, but not with N1-mutants (the xQuest software was used to search for cross-linked mono-, di-, tri- and tetra-nucleotide adducts and the data was filtered for mononucleotides that are present in FOXRBE mutants which are colour coded (*N indicates a $^{13}$C-labelled nucleotide, mutated nucleotides are labelled in red) (see Supporting Data 1). d SPR sensorgrams show that U or G mutations at N3, N4, N5 and N7 of FOXRBE attenuate, but do not abolish, FOXRBM binding to FOXRBM variants. e SDS-PAGE gels show that FOXRBM variants cross-link to FOXRBM with increasing irradiation (repeated three times). f CLIR-MS plots of singly-labelled FOXRBM variants show that protein-RNA cross-linking does not occur with U or G nucleotides located at N3, N4, N5 and N7 (see Supporting Data 1). (The xQuest software searches for cross-linked all mono-, di-, tri- and tetra-nucleotide that are present in FOXRBM mutants, and the data was filtered for mononucleotides which are colour coded).
been expected to cross-link in the wild type FOXRBE-FOXRRM interaction (Fig. S7). Together, the data obtained from these RNA- and protein mutants suggests that π-stack interactions between aromatic amino acids (e.g. phenylalanine, tyrosine or histidine) and guanosines or uridines are an important prerequisite for their cross-linking, not only to the aromatic side chains, but also to the flanking amino acids. Clearly, our findings do not speak to all cross-linking reactions in RNA-protein complexes, for instance those involving sulfur-containing amino-acids, such as cysteine, which is not present in the FOXRRM, but which is prone to cross-link probably due to the high reactivity of the thyl radical\(^{18,30,43}\).
RNA-protein cross-linking correlates with π-stacking interactions in other complexes. In order to determine whether these findings apply more broadly to RNA-protein cross-linking, we examined CLIR-MS data from the alternative splicing factor PTBP1 in complex with the internal ribosomal entry site (IRES) of encephalomyocarditis virus (EMCV)28. Cross-links mainly clustered around five aromatic amino acids (Y127, Y267, H411, H457), and comprised uridines, as reported by ref. 28. Correlating these observations with the NMR solution structure of PTBP1 bound to short poly-pyrimidine sequences (CUCUCU)44, confirmed that these amino acids were indeed all π-stacked to pyrimidines (PDB IDs: 2AD9, 2ADB, 2ADC) (Fig. S8), with cross-linking extended for a few amino acids along the protein backbone, flanking the aromatic side chains. Tyrosines Y127, Y267 and histidine H411 π-stack to uridines in CUCUCU, consistent with uridine-containing cross-links in the CLIR-MS dataset from the IRES of EMCV (Fig. S8b). Intriguingly, however, histidine H457 π-stacks to cytidines in the IRES28 and CUCUCU44, but produces high numbers of U- and UU-containing cross-links in the CLIR-MS dataset28. Likewise, Gléry et al observed by NMR spectroscopy a π-stacking of cytidine to Y19 in the RM of SRIF145, whereas Kramer et al. found a uridine cross-linked to the same amino acid18. These observations suggested that C-to-U conversion (i.e. hydrolysis) might occur at π-stacked cytidines during cross-linking or in sample work-up/analysis. Although the cytosine group itself is stable to the conditions used to fragment RNA by base hydrolysis or enzyme digestion (refs. 46,47), the exocyclic amino group of cytidine is more susceptible to hydrolysis when its 5–6 double bond is reduced, i.e. in dihydrocytidine48,49 (Fig. S6b). Since cross-linking reactions may produce intermediates or final products in which the cytidine 5–6 carbon-carbon bond is saturated, it is plausible that C-to-U conversion only occurs at π-stacked/cross-linked cytidines. Hence, mindful of the minor differences in the masses of cytidine/uridine-containing fragments, we searched our datasets for supporting evidence of this, using an appropriate set of parameters for the xQuest software. We did not observe significant 13C-to-13U hydrolysis using CLIR-MS on UG*CAUGU and the FOXRBE mutant *CGCAGU. However, this might have been because neither of these cytidines underwent efficient cross-linking/π-stacking to FOXRBE (UG*CAUGU: Fig. 1c, third panel; *CGCAGU: Fig. S5, second panel). Therefore, we also analyzed additional CLIR-MS data from four fully 13C-labelled FOXRBE mutants bearing cytidines at positions N4, N5, N6, and N7 (Fig. S6c). Indeed, we found that two of the mutants (UGCGACGU and UGCAUCGU) produced large numbers of cross-links that - consistent with the NMR structure - could only have derived after C-to-U conversion; for example, AU and AUGU from UGCGACGU, bound to H120; and AUU and UUU from UGCAUCGU, Bound to F160 (Fig. S6c–f). Taking together the data from the PTBP1 study, that of SRSF118,45 and that of these six FOXRBE RNAs, we concluded that cytidine likely undergoes partial hydrolysis mainly at positions in an RNA where it π-stacks and cross-links to the protein; for FOXRBE, at positions N5 and N6, but not at positions N3, N4, and N7. In contrast to previous assumptions15,17, these findings provide direct mass-spectrometry evidence that cytidine in RNA-protein complexes readily participates in photo-induced cross-linking, especially when it is π-stacked. However, this renders it susceptible to hydrolysis to uridine, which confounds its detection and in some cases may even lead to mis-assignments during RNA-protein modeling.

Next, we sought to confirm the importance of π-stacking to RNA-protein cross-linking in datasets that were generated using techniques other than CLIR-MS. Thus, we searched for structurally well-characterized examples in literature that would speak to the generalization of our findings. A unique strength of the CLIR-MS technique is that in many cases it is possible to simultaneously identify both the precise amino acid and the ribonucleotide in a cross-linked fragment. Indeed, we identified only one published example where cross-linking at both the ribonucleotide and the amino acid were unambiguously defined by isotopic labelling, and where these sites could be mapped to a high resolution structure. In this case, 18O-RNA labelling and targeted mass spectrometry were used to localise the cross-link of U11 in a let-7 microRNA to a π-stacked phenylalanine (F55) in the LIN28 cold shock domain (Fig. 3e)50–52. On the other hand, we found numerous examples where amino acids involved in stacking interactions (predominantly with uracils) underwent UV-cross-linking, most likely with the same uracil but not unambiguously proven by nucleotide labelling. For example, Panhale et al. report a cross-link between F19 and a uridine in hnRNPC, from which the NMR structure with poly-U sequences confirms the π-stacking interaction with F1953,54 (Fig. 3d). Kramer et al. used a sophisticated workflow to pin-point-cross-linking sites on a broad scale from ribonuclear protein complexes (RNPs) isolated from human and yeast cells18. By correlating their cross-linking data from ribosomal yeast protein S1 with the crystal structure of the protein (PDB ID: [4V88]) (Fig. 3e)55, we confirmed that tryptophan W117 π-stacks and cross-links to uridine U1799 from ribosomal S1. Similarly, the same group localised RNA cross-links on the human splicing factor U2AF65-KdA subunit to amino acids L261, F262 and F199; according to the crystal structure, F262 and F199 both π-stack to uridines in complex with poly-U RNA (Fig. 3f, g) (PDB ID: 2YHI)56. Bae et al. showed that tyrosines Y325 (Fig. 3h), Y450 and Y1356 in the Streptococcus pyogenes Cas9 (spCas9) protein all cross-link with RNA17; the crystal structure of spCas9 shows that all three residues are π-stacked to uridines or guanosines57.

These well-characterized, selected examples already provided supporting evidence for the generality of our findings. However, the aforementioned examples of Kramer et al. and Bae et al. were
extracted from large well annotated datasets in which, collectively, more than 100 RNA-protein cross-links from a wide variety of RBPs are catalogued. These datasets therefore offered an opportunity to analyze in an unbiased fashion the putative link between π-stacking and cross-linking. The two proteome-wide datasets reported by Kramer et al. each comprise approximately 60 RNA-protein cross-links generated from affinity-captured nuclear pre-mRNAs from human cells18 and from pre/mRNAs of yeast cells18. The third dataset reports 84 cross-links to spCAS9, which forms a complex with single guide RNAs17. We manually annotated each of the three datasets in a systematic fashion in order to determine whether amino acids that undergo cross-linking are located within +/− 3 positions of an aromatic amino acid side chain (mindful that in a fully random sequence, 20% of the amino acids may be aromatic) and if yes, whether said aromatic side-chains π-stack to nucleobases.

The human RNP dataset18 details 60 cross-links to approximately 35 proteins, with 37 cross-links that are localized on defined amino acids, in mostly RRM binding domains (Supporting Data 4). From these, 33 cross-links are assigned to non-cysteine and non-methionine amino acids (Fig. 3i; Supporting Data 4), 29 of which are located within three amino acids of an aromatic side chain. High-resolution structures were informative for 19 of these amino acids and, pleasingly, showed that 18 of the aromatic side chains were involved in apparent π-stacking interactions, and one which was not. Taking into account also the four cross-links which are not close to an aromatic amino acid, means that 18/23 (78%) cross-links occur close to a π-stacked aromatic side chain, fully consistent with our findings. In this dataset, neither of the KH domain-bearing proteins carry aromatic amino acids close to cross-links, although both underwent cross-linking to cysteines, demonstrating that cysteine does not follow the pattern, as expected. In contrast, a positive π-stacking/cross-linking association (to an adenosine) was present for the cold shock domain of Y-box binding protein, as well as for ribosomal proteins S2, L5, L6 and L34 with distinct domains.

The yeast RNP dataset18 contains 39 defined cross-links to 52 proteins, containing a variety of domains (Supporting Data 4). Surprisingly, 23/39 cross-links involve cysteines, which the authors suggested might be due to the present of dithiothreitol (DTT) in the yeast sample which is known to promote cross-links involving cysteine residues18,58. Fourteen cross-links lie within three amino acids of an aromatic side-chain, for which 12 high-resolution structures are available. These show that six cross-links occur at apparent π-stacking interactions, and for two cross-links high-resolution structures are not available (Fig. 3i; Supporting Data 4). Finally, the outcome of cross-linking of the spCAS9 protein to RNA was reported by ref. 17. Cross-links comprising 40 amino acids were catalogued, of which five were cysteine or methionines and were discarded from further analysis. Of these 35 cross-links, 32 lie within three amino acids of an aromatic side chain and of these, 20 can be studied with the high-resolution structure. Twelve of the cross-links involve apparent π-stacking interactions (Fig. 3i; Supporting Data 4), whereas eleven cross-links are not close to a π-stacking interaction. In summary, this unbiased analysis confirmed the association of cross-linking with π-stacking in a variety of RNA-binding domains for totals of 78, 42 and 52% of the cross-links in studies performed by independent groups in yeast, bacterial and human systems.

Taken together with the aforementioned specific examples from literature and our analysis of the FOXRRM and PTBP1 CLIR-MS data, the data overall strongly supports the importance of π-stacking to the RNA-protein cross-linking chemistry. The absence of a positive correlation for some cross-links may be due to a variety of reasons; e.g. different conditions for protein structures/domain determination in vitro and cross-linking experiments performed in vivo on protein complexes; or cross-linking reactions that occur as a result of transient interactions (i.e. artifacts). In addition, the lack of structural information for several cross-links in the human RNP and spCAS9 datasets may have prevented an even higher correlation. Finally, it is also apparent that a π-stacking interaction is not a strict requirement for all cross-linking events. Cysteine, which is prone to cross-linking, does not require a π-stacking interaction in order to produce a long-lived, highly reactive radical18,30,43. This is consistent with the lack of aromatic amino acids proximal to cysteine-containing cross-links in the KH domains of proteins in the yeast and human RNP data-sets18. Thiol-containing molecules present in buffer may also initiate UV-induced cross-linking of proteins and nucleic acids59. In addition, recent publications described cross-linking of dsDNA to histones using conventional cross-linking59, where π-stacking of the side chain is more difficult to envision because of the double-stranded helical structure; although this may partly explain why double-stranded oligonucleotides are reported to cross-link less efficiently than single-stranded oligonucleotides60,61.

Photo-induced electron transfer in a π-stacked RNA-protein complex may mediate radical reactions of cross-linking. Free radical reactions of nucleic acids and proteins have been well studied in the context of oxidative damage and electron transfer43,62, but less thoroughly investigated for RNA–protein interactions29,41. However, a description of the photo-induced intramolecular cyclization of 5-benzyluracil and 6-benzyluracil via benzyl and uracil radical intermediates suggests a plausible model for the cross-linking of U1 with F126 (Fig. 4a)35. Hence, photo-induced electron transfer between U1 and F126 generates a short-lived anion/cation radical pair (exciplex) (Fig. 4b; structures 1 and 2). Subsequent protonation of the uracil radical anion can yield a neutral α-hydroxy radical43, whereas ready deprotonation of the F126 radical cation will produce a stabilized benzylic radical. In the absence of oxygen, the major fate of these free radicals is recombination with the formation of the direct U1–F126 cross-link (Fig. 4b; structure 4). An analogous mechanism has been proposed for the reaction between uracils/halogenated uracils and tyrosine derivatives31,63.

Alternatively, the F126 radical, or radical cation, may rearrange to neighboring amino acids in processes mediated by hydrogen atom abstractions43, or via oxidation of amide carbonyls (by the F126 radical cation)64, yielding free radicals at peptide α-carbon sites on the protein backbone. Viehe et al have proposed that α-carbon radicals are especially stabilized thermodynamically by capto-dative effects, i.e. simultaneously by electron-withdrawing (−C = O) and electron-donating (−NR2) groups65 and, furthermore, that they readily combine with other radicals.

Hence, depending on the lifetimes and the locations of these radicals on the protein backbone, “indirect” cross-links to U1 may form, yielding products that are identified by mass spectral analysis after controlled digestion (e.g. structures 5–7; Fig. 4b). These steps are consistent with the outcome of cross-linking reactions of the F126 mutants. Thus, the exchange of phenylalanine for histidine and tyrosine produced similar direct and indirect cross-links, whereas leucine was mostly inactive since its aliphatic side chain cannot partake in the initial electron transfer. Several efforts to mimic some of these cross-linking reactions in solutions were unsuccessful, confirming the crucial role played by the local protein binding site environment. Based on the similarity of the cross-linking profiles from U1 and G1 (Fig. 1c), it seems intuitively likely that guanosines G2 and G3 may follow a similar mechanistic reaction path as U1. Thus, photo-excitation of the stacked guanine-phenyl ring systems produces free radicals at
G2 and G6, as well as on the peptide backbone around F126 and F160. Recombination yields direct and indirect cross-links, which in the case of G2 are to the same α-carbon radicals that couple with U1. The nature of the initial exciplex formed from electron-transfer in a stacked guanosine-phenylalanine is unclear, and we were unable to identify a literature precedent for such a mechanism. However, well-cited studies have shown photo-induced electron transfer between π-stacked pyrimidine and purine nucleobases that produce long-lived exciplexes.66,67. Electron transfer between an amino acid and a nucleotide might be expected to occur in the direction that yields the lowest-energy exciplex. However, due to the special environment of an RNA-protein binding site (see discussions in refs. 64,66), this may not necessarily correlate with the measured redox potentials of isolated nucleotides or aromatic amino acid side chains. Together, our observations demonstrate the importance of local environment to cross-linking in the RNA-protein binding site, and at least partly explain why cross-links occur only at specific positions in an RNA-RBP motif.

Discussion

For a complete understanding of the roles that RBPs play in cellular processes, it is necessary to understand at the atomic level how RNA binding domains in proteins engage with RNAs. RNA-protein interactions are generally characterized in two main ways in vivo: isolating proteins and sequencing the bound RNAs (CLIP methods), and identifying proteins bound to RNAs, for example, by mass spectrometry. Most of these approaches rely upon photo-induced cross-linking, which provides direct evidence of binding under native conditions. However, presently, native cross-linking-based methods suffer from two drawbacks: i) it is challenging to identify simultaneously sites of cross-linking on the RNA and protein, ii) cross-linking in an RNA-RBP motif typically proceeds inefficiently and in an unpredictable fashion. Therefore, any progress that further us our understanding of this chemistry is of high value.

The CLIR-MS method employs isotope-labelled RNAs to resolve amino acid/ribonucleotide cross-links in a single protocol, whereby segments of labelled RNA are produced by in vitro transcription prior to ligation-assembly into a full-length RNA. In this study, we have broadened the application of CLIR-MS through the use of chemically synthesized 13C-labelled RNAs. This enables site-specific incorporation of labelled nucleotides into the RNA. After irradiation of the RNA-protein complex, and controlled digestion to nucleotide-peptide adducts, the locations of cross-linked nucleotides are pinpointed site-specifically. We demonstrated this methodological advance with a study of the interaction of the RRM domain of the RBFOX family bound to its consensus binding element (U)GCAUGU, for which we have previously determined an NMR structure and studied cross-linking. Photo-irradiation of the FOXRRM-FOXRBE complex led to key observations with potentially wide-ranging implications: 1) strong cross-linking occurred between U1, G2 and G6 with clusters of amino acids centred around the phenylalanines F126 and F160; 2) very little cross-linking was observed at other uridines in the parent or a mutated FOXRBE and 3) amino acids that flank F126 and F160 also cross-linked efficiently to U1, G2 and G6, but not to other nucleotides of FOXRBE. Since the NMR structure of FOXRRM-FOXRBEP36 shows that U1 and G2 π-stack to F126, and that G6 π-stacks with F160, the data suggested that a π-stacking interaction is a requirement for cross-linking events in an RNA-protein interaction, at least for this RRM domain. Indeed, other aromatic side chains could substitute for F126 in cross-linking, but incorporation of leucine abolished direct and almost all indirect (flanking) cross-linking to U1/G2. Other researchers have noted in passing the increased presence of aromatic amino acid side chains in UV cross-linking datasets (see refs. 17,18,29,52,68), but have not to our knowledge recognized its role as a trigger for cross-linking, nor distinguished between direct and indirect cross-link events. We validated our results on the RBFOX system with the correlation of published cross-linking and structural data from CLIR-MS data generated with the PTBP1 protein, and selected examples from LIN28, hnrNPC, U2AF, ribosomal yeast protein S1 and bacterial spCAS9, that were produced using different methods. Our findings were further strengthened by an unbiased analysis of more than 100 cross-links in large-scale data-

Fig. 4 Proposed mechanism for UV induced RNA-protein cross-linking. a UV induced cyclization of 5-benzyluracil and 6-benzyluracil after Sun et al.35. (bonds in red are formed upon cyclisation). b Possible mechanism of UV cross-linking between the stacked F126 und U1 of the FOXRRM (1). Photo-induced electron transfer leads to a radical ion pair (2). After protonation/deprotonation steps, the radicals on the benzylic position of F126 and C4 of uridine (3) recombine to yield direct cross-links (4). Indirect cross-links between U1 and R127 (5), P125 (6) or I124 (7) may form when the radical cation of F126 oxidizes amide carbonyls from flanking amino acids, which rearrange to radicals stabilized by capto-dative effects at the α-carbons (*)
sets comprising various RNA-binding domains, where in one case up to 78% of the cross-links showed π-stacking to a proximal aromatic amino acid side chain. It is clear that factors in addition to π-stacking also contribute to cross-linking events in RNA-protein sites, including efficiency of the photo-induced electron transfer between nucleobase and amino acids, the ability to stabilize free radicals, the flexibility of the structure to adopt to the configurations that are required for the radical reactions and the proximity of reacting radical pairs. Furthermore, our findings do not explain all RNA-protein cross-linking reactions, including those involving cysteine, which is highly photoreactive and prone to cross-link probably due to the high reactivity of the thyl radical.

The major findings in this study were enabled by the combination of site-specific labeling with the CLIR-MS protocol, which together provides enhanced knowledge of cross-linking sites at single-nucleotide and amino acid resolution. These included the surprising discovery that cytidine residues which are π-stacked to aromatic residues can undergo partial hydrolysis during photo-induced cross-linking. This observation may explain discordance in some cases between structural- and cross-linking data. Furthermore, the hydrolysis of cytidine should be anticipated in the analysis of CLIR-MS data and may also be relevant to the interpretation of data from CLIP experiments, which is currently an area of intense activity.

CLIR-MS technology is inherently flexible and we are exploring further improvements to the method. However, the method described here requires the use of chemically synthesized, isotope-labelled RNA and is currently restricted to the study of purified individual RNA-protein complexes. Nevertheless, the data produced can aid the interpretation of that from unbiased complex systems. For instance, our findings extended the knowledge on the role of the local environment to cross-linking in the binding site, i.e. beyond the simple proximity of photo-reactive nucleotides and amino acids. This helps at least partly to explain why cross-links occur only at specific sites in an RNA-RBP motif. Furthermore, the localization of π-stacking interactions will aid the interpretation of proteome-wide datasets, for example in cases where proteins lack canonical RNA-binding domains, and in the analysis of CLIP datasets. Thanks to the inherent variations in the ways that RBPs recognize their RNA targets, predictive modeling of RBP selectivity is extremely challenging; our findings can be implemented into the development of new tools for de novo motif discovery.

In a broader sense, the RNA-binding sites of RBPs have garnered attention in the context of disease and drug targeting; for example, the RNA binding site in the intrinsically disordered region of TDP43 contributes to its aggregation in amyotrophic lateral sclerosis (see ref. 17). A fuller understanding of how RNA binding domains in proteins engage with RNAs can support the development of new methods of targeting RBPs via the RNA binding site.

**Methods**

**Protein expression and purification.** The FOXRBM and its mutants were expressed in transformed BL21 Codon+ Escherichia coli at 37 °C in LB medium with kanamycin and chloramphenicol. The cells were induced with 1 mM IPTG and after 4 h the cells were harvested by centrifugation. Cells were lysed in 50 mM Na2HPO4, 1 M NaCl, pH=8 using a cell cracker and the cell lysate was centrifuged at 17,000 rpm at 4 °C for 30 min. The supernatant was purified using a NiNTA affinity column (Ni-NTA agarose, Qiagen). After washing with buffer 50 mM Na2HPO4, 3 M NaCl, pH=8, the protein was eluted with a step gradient of imidazole (40–500 mM). The purest fractions as judged by 5–20 % SDS-PAGE were combined, and the column was repeated. Pure fractions were dialyzed against 5 L 20 mM NaCl, 10 mM NaH2PO4, pH=6.5 overnight. The identity of the FOXRBM and its mutants was confirmed using LC-MS/MS measured by top-down analysis and data analysed using TopPIC.

For the biotinylated FOXRBM, the 15- amino acid E. coli biotin ligase recognition sequence GGDNFEAQKEWHE was introduced between the TEV cleavage site and an encoding FOX-1 mutant PCR amplicon. E. coli protein ligase BirA was cloned, expressed and purified as previously described. The generation of biotinylated FOXRBM was achieved in a 10 mL batch-mode cell-free synthesis reaction which was conducted for 3.5 h in the presence of 2 µM BirA and 400 µM D-biotin. The proteins were purified as described above and the biotinylated proteins were cleaved overnight at 4 °C with 0.5 mg TEV protease.

**RNA synthesis.** The synthesis of all oligonucleotides was carried out with the MM12 synthesizer (Bio Automation Inc., Plano, TX) on a 50 nmol scale using 500 A UnyLinker CPG using standard conditions. Synthesis conditions, purification methods and characterisation (Supplementary Table 1, Fig. S10) are listed in supplementary methods.

**Surface Plasmon Resonance Spectroscopy (SPR).** The SPR analysis was carried out on the MASS-1 or SPR-2 from Sierra Sensors (Hamburg, DE). For coating, the amine chip was first treated with PBS buffer at a flow rate of 12.5 µl/min at a pH of 7.5. Next, a solution of 1 M NaCl and 1 M NaOH was injected to all 16 channels for 2 min. Afterwards, 100 µl of a mixture of 200 mM EDTA and 100 mM NHS was added. For coating of the streptavidin, an acetylate buffer (10 mM sodium acetate) at a pH of 5.5 was used and a 100 µl injection resulted in an approx. response of 2500 RU. The running buffer was switched to a HEPES buffer (10 mM HEPES at pH 7.4, 200 mM NaCl, 3.4 mM EDTA, 0.01 % (v/v) Tween 20) before capturing the analyte. Approximately 10 µl of a 75 nM solution of biotinylated FOXRBM in HEPES buffer was injected only on the second channel resulting in a response of approx. 200 RU. The amount of the injected ligand varied depending on the desired coating. 100 µl of the analyte was injected at a flow rate of 25 µl/min with a dissociation time of 480 s. For regeneration, 50 µl of a 2 M NaCl solution was used. After every injection, a buffer injection was added for double referencing. The binding affinities were determined from kinetic measurements or using steady-state measurements.

**Cross-linking and gel electrophoresis.** Complexes of FOXRBM and the desired RNA were prepared by mixing both components in equimolar ratios at the desired concentration of 10 mM in 10 mM sodium phosphate (pH = 6.5) and 50 mM NaCl and incubated for 10 min on ice. 15 µl of the sample solutions were placed in a 96 well plate on ice and irradiated at 1000 μJ/cm2. For coating of the streptavidin, an acetate buffer (10 mM sodium acetate) at a pH of 5.5 was used and a 100 µl injection resulted in an approx. response of 2500 RU. The running buffer was switched to a HEPES buffer (10 mM HEPES at pH 7.4, 200 mM NaCl, 3.4 mM EDTA, 0.01 % (v/v) Tween 20) before capturing the analyte. Approximately 10 µl of a 75 nM solution of biotinylated FOXRBM in HEPES buffer was injected only on the second channel resulting in a response of approx. 200 RU. The amount of the injected ligand varied depending on the desired coating. 100 µl of the analyte was injected at a flow rate of 25 µl/min with a dissociation time of 480 s. For regeneration, 50 µl of a 2 M NaCl solution was used. After every injection, a buffer injection was added for double referencing. The binding affinities were determined from kinetic measurements or using steady-state measurements.

**Cross-linking and mass spectrometry.** 75 µg of RNA-protein complexes were made of equimolar mixtures of unlabelled and 15C-labelled RNA and irradiated four times with 800 µl/cm2 as described above. Each irradiation step was separated by 1 min for sample cooling. After irradiation, samples were precipitated with 3 volumes of ethanol at −20 °C and 1:10 volumes 3 M sodium acetate (pH 5.2), left at −20 °C for at least 2 h, and centrifugated at 4 °C for 30 min at 13,000 × g. Resulting pellets were washed by brief vortexing in 80% ethanol at −20 °C, and centrifugation was repeated. For the digestion with alkaline hydrolysis: Pellets were air dried for 10 min, then were resuspended in 50 µl of 50 mM Tris–HCl (pH 7.9), 1 ml 0.1 M NaOH was added, and the sample incubated at 70 °C for 10 min on a shaking incubator. The sample was neutralized with 105 µl 1 M HCl, cooled on ice, purified using solid-phase extraction and evaporated to dryness in a vacuum centrifuge. The sample was resuspended in 50 µl 50 mM Tris–HCl, pH 7.9, 4 M urea and then diluted with 150 µl 50 mM Tris–HCl, pH 7.9. The exact procedures of the digestion using RNases and trypsin, the enrichment using titanium dioxide affinity chromatography, and LC-MS analysis are described in the Supplementary Information. All identified cross-links are listed in Supporting Data 2 and the masses of the RNA adducts and neutral mass losses are given in Supporting Data 3.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE77 partner repository with the dataset identifier PXD031381. The referenced accession codes for the structures in the Protein Data Bank are 2ERR, 2AD9, 2ADB, 2ADC, 3TS0, 2MXY, 4V88, 2YH1, 4ZT0.

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Author contributions

A.K. and T.d.V. expressed and purified the RBFOX RRM for the study. M.S. performed the SPR studies. A.K. produced chemically synthesized RNA and performed cross-linking of protein-RNA complexes. A.K. and C.S. performed sample preparation for mass spectrometry and analysed the data using xQuest. All authors (A.K., C.S., T.d.V., M.S., M.G., R.A., F.A., A.L., J.H.) interpreted the data. A.K. and J.H. wrote the manuscript together. All authors (A.K., C.S., T.d.V., M.S., M.G., R.A., F.A., A.L., J.H.) contributed to manuscript revisions and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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External validation

Please provide the validation method used to verify the accuracy of the results. This could include any statistical tests, cross-validation techniques, or other methods used to confirm the reliability of the findings.