Staufen1 dimerizes through a conserved motif and a degenerate dsRNA-binding domain to promote mRNA decay

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Staufen1 (STAU1)-mediated mRNA decay (SMD) degrades mammalian-cell mRNAs that bind the double-stranded RNA (dsRNA)-binding protein STA1 in their 3′ untranslated region. We report a new motif, which typifies STA1 homologs from all vertebrate classes, that is responsible for human STA1 (hSTAU1) homodimerization. Our crystal structure and mutagenesis analyses reveal that this motif, which we named the Staufen-swapping motif (SSM), and the dsRNA-binding domain 5 (‘RBD’5) mediate protein dimerization: the two SSM α-helices of one molecule interact primarily through a hydrophobic patch with the two ‘RBD’5 α-helices of a second molecule. ‘RBD’5 adopts the canonical α-β-β-α fold of a functional RBD, but it lacks residues and features required to bind duplex RNA. In cells, SSM-mediated hSTAU1 dimerization increases the efficiency of SMD by augmenting hSTAU1 binding to the ATP-dependent RNA helicase hUPF1. Dimerization regulates keratinocyte-mediated wound healing and many other cellular processes.

In mammals, STA1 mediates embryonic stem cell differentiation1, mRNA transport and localization2-3, mRNA translational activation4, HIV type 1 assembly5,6 and SMD7-10. During SMD, STA1 triggers the translation-dependent degradation of specific mRNAs that contain a STA1-binding site (SBS) within their 3′ untranslated region (3′ UTR) to regulate gene expression during myogenesis7, keratinocyte motility10, adipogenesis11 and probably other mammalian cellular pathways. In human cells, SBSs can be created in cis by intramolecular base pairing within an mRNA 3′ UTR9 or in trans by base pairing between partially complementary Alu elements within an mRNA 3′ UTR and a long noncoding RNA10. When translation terminates sufficiently upstream of an SBS so as not to disrupt the SBS, association of the UPF1 RNA helicase with SBS-bound STA1 triggers mRNA decay (reviewed in ref. 12).

Generally, similarly numbered STA RBDs from different species are more identical than are differently numbered RBDs within the same protein13, suggesting a common overall design of RBDs in STA1 homologs. hSTAU1 has 496- and 577-residue isoforms (hSTAU155 and hSTAU163, respectively; NCBI Gene ID 6780), each of which contains RBD2, RBD3 and RBD4, and only the N- and C-terminal regions of what would be hSTAU1 ‘RBD’5 (ref. 18); additionally, hSTAU256 and hSTAU262 have a complete RBD1, whereas hSTAU252 and hSTAU259 contain a truncated RBD1 (refs. 3,18,19). Similarly to hSTAU1, hSTAU2 mediates not only mRNA decay20 but also mRNA localization21. Each paralog and even some of their isoforms may function and localize differently within cells3,19,21.

The three-dimensional analyses of STA1 proteins have been limited to two RBD structures. The first is the NMR structure of Drosophila melanogaster STA1 RBD3 bound to a 12-bp stem-loop RNA, which revealed the interaction of the canonical α-β-β-β-α fold with dsRNA22,23. The second is of mouse STA2 RBD4 in the absence of dsRNA (Protein Data Bank (PDB) ID 1UHZ; RIKEN Structural Genomics Initiative), which also showed the α-β-β-β-α fold. In general, evidence for structure- or sequence-specific recognition of cognate RNAs by RBDs remains elusive. RBD1 and RBD2 of the mouse adenosine deaminase ADA2 recognize distinct bases within a human pre-mRNA GluR-2 (GRIA2) stem loop because of subtle sequence and structural differences in their RNA-interacting regions24. However, what hSTAU1 recognizes when it binds dsRNA remains unknown.

A recent study25 using cultured cells demonstrated that multiple hSTAU155 molecules can bind to the SMD target encoding human ADP ribosylation factor 1 (hARF1)9. Using yeast two-hybrid analyses, the authors identified a region in ‘RBD’2 and a region containing ‘RBD’5 that separately interact with full-length hSTAU155; studies in

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cultured cells showed ‘RBD’5 seemed to mediate the stronger interaction\textsuperscript{25}. We recently discovered that some SBSs consist of intermolecular duplexes of partially complementary Alu elements that range from 86 to 298 nucleotides\textsuperscript{10} and might support the binding of more than one hSTAU1 molecule. These amino acids consist of the C terminus of hSTAU1\textsuperscript{55} and include ‘RBD’5 (Fig. 1a and Supplementary Fig. 1a), which has only 18\% sequence identity to the prototypical hSTAU1 RBD3 and does not bind dsRNA\textsuperscript{15,17}. Multiple sequence alignments, carried out using ClustalW\textsuperscript{26}, of full-length hSTAU1 with hSTAU2 and STAU orthologs from representatives of the five major vertebrate classes revealed a conserved sequence residing N-terminal to ‘RBD’5 that consists of hSTAU1\textsuperscript{55} residues 371–390 (Supplementary Fig. 1a). We named this motif the SSM (Fig. 1a and Supplementary Fig. 1a) for reasons explained below. Despite an identifiable ‘RBD’5, an SSM is absent from, for example, \textit{D. melanogaster} and \textit{Caenorhabditis elegans} STAU proteins (Supplementary Fig. 1b). However, STAU proteins in other invertebrates contain both SSM and ‘RBD’5 regions (Supplementary Fig. 1b). The SSM is proximal to the TBD, which spans residues 282–372 (ref. 15) (Fig. 1a), and it overlaps with residues 272–405, at least part of which recruits hUPF1 during SMD\textsuperscript{7}.

Structure of hSTAU1 SSM–‘RBD’5

A search of the NCBI Conserved Domain Database\textsuperscript{27} did not identify hSTAU1 ‘RBD’5 as an RBD. To understand the atomic details of SSM–‘RBD’5, we purified hSTAU1 residues 367–476 from \textit{Escherichia coli} (Supplementary Fig. 2a), produced crystals that we verified were intact using SDS-PAGE and silver staining (Supplementary Fig. 2a), and solved its X-ray crystal structure at 1.7 Å (Table 1). Our structure revealed that ‘RBD’5 adopts the α-β-β-α topology of a prototypical RBD, and the SSM forms two α-helices (hereafter called SSM α1 and α2) that are connected by a tight turn (Fig. 1b and

Figure 1

**Comparison of vertebrate STAU sequences, and the X-ray crystal structure of hSTAU1 SSM–‘RBD’5.** (a) Modular organization of hSTAU1\textsuperscript{55}. Relative positions are indicated for the true RBDs RBD3 and RBD4, degenerate ‘RBD’2, the TBD \textit{in vitro} (residues 282–372) and the SSM–‘RBD’5 region (residues 367–476). The secondary structure of hSTAU1\textsuperscript{55} (blue) derives from data reported here, and the dashed line denotes residues 397–402 that are missing from the crystal structure. (b) X-ray crystal structure shown as a single hSTAU1\textsuperscript{55} SSM–‘RBD’5 molecule (left, blue) and a domain-swapped dimer formed with another molecule in the crystal lattice (right, green). Asterisks denote alternative conformations of the linker (Supplementary Fig. 2c). Connectivities between an SSM and ‘RBD’5 that could not be modeled because of multiple conformations and/or disorder in the crystal structure are indicated by dotted lines. Structural elements are also identified. (c) Close-up view of the interaction between the SSM α-helices (green, α1 and α2) and the ‘RBD’5 α-helices (blue, α1 and α2). Important residues are shown as stick representations and are labeled. Residues that contribute to the hydrophobic core are yellow. Hydrogen bonds between polar atoms are shown as dashed lines. (d) Polar interactions between SSM Arg376 and ‘RBD’5 main chain oxygens; the SSM is green, ‘RBD’5 is blue, and key residues and the citrate ion ligand (orange) are shown as sticks and are labeled. Hydrogen bonds are shown as dashed lines.
Table 1 Crystallographic data collection and refinement statistics

|                      | Ethyl-mercury SADa | Native 1a | Native 2a |
|----------------------|--------------------|-----------|-----------|
| **Data collection**  |                    |           |           |
| Space group          | P4_122             | P4_122    | P4_122    |
| Cell dimensions      |                    |           |           |
| a, b, c              | 47.4, 80.9         | 48.5, 82.1| 45.7, 86.1|
| Resolution (Å)b      | 25.81–2.92 (2.94–2.92)| 50–2.20 (2.24–2.20) | 50–1.70 (1.76–1.70) |
| R_symb               | 34.2 (69.0)        | 6.9 (42.7)| 8.5 (26.3)|
| I / αI              | 11.2 (3.4)         | 34.4 (6.7)| 28.9 (10.6)|
| Completeness (%) b   | 99.6 (96.7)        | 99.5 (100.0)| 99.9 (99.7)|
| Redundancyc          | 10.5 (3.5)         | 19.3 (20.0)| 13.8 (13.3)|
| **Refinement**       |                    |           |           |
| Resolution (Å)       | 31.35–1.70         |           |           |
| Number of reflections| 10,601             |           |           |
| Rwork / Rfree       | 16.3 / 20.2        |           |           |
| Number of atoms      |                    |           |           |
| Protein              | 864                |           |           |
| Ligand/ion           | 14                 |           |           |
| Water                | 63                 |           |           |
| B factors            |                    |           |           |
| Protein              | 27.5               |           |           |
| Ligand/ion           | 46.0               |           |           |
| Water                | 33.9               |           |           |
| r.m.s. deviations    |                    |           |           |
| Bond lengths (Å)     | 0.011              |           |           |
| Bond angles (°)      | 1.49               |           |           |

Data set was obtained from a single hSTAU1 SSM-'RBD'5 crystal. aHighest-resolution shell values are in parentheses. SAD, single-wavelength anomalous dispersion.

Supplementary Fig. 2b). Electron density was clearly interpretable for the SSM and 'RBD'5 but not for residues 397–402, which form part of the linker (residues 393–406) between the SSM and 'RBD'5.

Pro408 (which starts α1), Leu412, Leu415 and Val418; and Phe421 of loop 1 (L1) (Fig. 1c). Additionally, 'RBD'5 α2 contributes Leu466, Leu469, Leu472 and Leu475 (Fig. 1c).

Figure 2 Comparison of hSTAU1S55 'RBD'5 with an RBD that binds dsRNA. (a) Superimposition of hSTAU1 'RBD'5 (green) and A. aeolicus RNase III RBD29 (gray) using Dali28. (b) Structure-based sequence alignment of hSTAU1S55 'RBD'5 (top, green sequence) and A. aeolicus RNase III RBD29 (bottom, gray sequence). Conserved amino acids are in orange boxes. Key residues discussed in the text are indicated with arrows. Structurally corresponding residues between the two proteins within region 3 are blue if positively charged and red if negatively charged. (c) X-ray crystal structure of A. aeolicus RNase III RBD in complex with dsRNA within a hairpin29. The protein is gray and in cartoon form, and the dsRNA is in stick representation. The three major dsRNA-interacting regions of typical RBDs29 are approximated in boxes numbered 1–3 to illustrate the important secondary structures. (d) Cartoon of the structure of A. aeolicus RNase III RBD bound to dsRNA (left) or STAU1S55 'RBD'5 superimposed on the structure of A. aeolicus RNase III RBD (right) and depicted in the same dsRNA binding configuration as the A. aeolicus RNaseIII RBD structure bound to dsRNA. Vacuum electrostatic potentials were generated using PyMOL to illustrate charge variance; on the surface representation of each protein, blue is positive, red is negative, and white is neutral. The three major dsRNA-interacting regions are boxed as in c.
Of the two polar interactions at the SSM-‘RBD’5 interface, one is a basic charge contributed by SSM Arg376: its two η-amine groups hydrogen bond with two carboxyl groups of the citrate anion present in the crystal structure, and its η- and ε-amines interact with the main chain oxygens of Glu474 and Ser473, respectively, which are positioned near the C terminus of ‘RBD’5 α2 (Fig. 1d). SSM Arg376 is conserved in all vertebrates analyzed except Danio rerio, in which the residue is asparagine, and Glu474 and Ser473 are invariant in vertebrates that contain the ‘RBD’5 α2 C terminus (Supplementary Fig. 1a). In the other polar interaction, the side chain hydroxyl group of SSM Thr371 and the main chain oxygen of Lys367 hydrogen bond with the amine group of ‘RBD’5 Gln419, and the ε-amine of Lys367 hydrogen bonds with the hydroxyl group of Gln419 (Fig. 1c). SSM residues lacking strict conservation (Met373, Tyr380, Gly381, Thr383 and Pro385) are positioned on the solvent-exposed side, opposite the interface that interacts with ‘RBD’5 (Supplementary Fig. 2d).

Comparison of ‘RBD’5 to an RBD that binds dsRNA

We were surprised that the three RBD structures identified by the Dali server as the most structurally similar to ‘RBD’5 bind dsRNA (Supplementary Table 1). Of the three, Aquifex aeolicus RNase III RBD29 provides the most complete comparison. A structure-based sequence alignment of this RBD with hSTAU1 ‘RBD’5 revealed that although the two structures are nearly identical, hSTAU1 ‘RBD’5 has a slightly shorter L1, an altered L2 and a longer L3 (Fig. 2a,b). Furthermore, hSTAU1 ‘RBD’5 lacks key residues that typify the three RNA-binding regions (regions 1, 2 and 3) of canonical RBDs23 and are present in the A. aeolicus RNase III RBD (Fig. 2b). The clearest differences are in regions 2 (within L2) and 3. hSTAU1 ‘RBD’5 L2, which does not extend as far as A. aeolicus RNase III RBD L2 (Fig. 2a) and thus may be unable to reach the minor groove of dsRNA, lacks a histidine residue that interacts with the dsRNA minor groove in the A. aeolicus RNase III RBD29 and true RBDs23 (Fig. 2c). The importance of an L2 histidine residue derives from studies of D. melanogaster STAU RB3 (Supplementary Fig. 3a), in which RNA binding was lost when the sole L2 histidine was changed to alanine.22 With regard to region 3, the positively charged residues in the A. aeolicus RNase III RBD that interact with the negatively charged phosphate backbone spanning the dsRNA major groove are negatively charged in hSTAU1 ‘RBD’5 and might actually repel dsRNA (Figs. 2b-d). Consistent with this view, D. melanogaster STAU RB3 (ref. 22) also maintains a basic charge in region 3 (Supplementary Fig. 3a,b).

Human SSM-‘RBD’5 homodimerizes in solution and in cells

Our crystal structure analyses raised the possibility that the SSM could mediate hSTAU1 dimerization by trans interactions with ‘RBD’5. Thus, we tested whether SSM-‘RBD’5 is sufficient to mediate dimerization of hSTAU1. After purifying glutathione S-transferase–tagged SSM-‘RBD’5 from E. coli and removing the GST tag, we found that SSM-‘RBD’5 migrated at the size of a dimer during gel filtration (Fig. 3a). Sedimentation velocity determinations using analytical ultracentrifugation confirmed that the average weight distribution of SSM-‘RBD’5 shifted to lower Svedberg values at lower concentrations (Fig. 3b). The best-fit model for SSM-‘RBD’5 derived from the three highest concentrations tested (0.0090 mg ml−1 root mean s.d. (r.m.s. deviation) fit to the model) was one of rapid monomer (1.32 ± 0.02/–0.03 S)–dimer (2.21 ± 0.01 S) equilibrium, where the dimer $K_d$ was 79 ± 9 μM. That purified SSM-‘RBD’5 assumes a dimeric solution state supports the existence of a trans, swapped interaction between the SSM of one hSTAU1 molecule and the ‘RBD’5 of another.

To determine whether the SSM mediates dimerization of full-length hSTAU1 in vivo, we transiently transfected human embryonic kidney 293T (HEK293T) cells with a mixture of two plasmids: (i) pEGFP-‘RBD’5, which produces monomeric enhanced GFP (eGFP)-tagged ‘RBD’5, and either pmRFP-SSM-‘RBD’5 or pmRFP-‘RBD’5, which produce monomeric red fluorescence protein (mRFP)-tagged SSM-‘RBD’5 or mRFP-‘RBD’5, respectively; or (ii) pEGFP-SSM-‘RBD’5 and either pmRFP-SSM-‘RBD’5 or pmRFP-‘RBD’5 (Supplementary Fig. 4a). The results of immunoprecipitations in the presence of RNase A using anti-GFP or, as a negative control, mouse IgG (mlG) revealed that dimerization cannot occur between two ‘RBD’5 molecules but can occur if one of the two ‘RBD’5 molecules contributes an SSM (Supplementary Fig. 4a; see Supplementary Note 1 for extended details and Supplementary Table 2 for immunoprecipitation and coimmunoprecipitation efficiencies).

To exclude the possibility that linker residues 393–406 contribute to the interaction between the SSM of one hSTAU1 molecule and ‘RBD’5 of another, we tested whether eGFP-SSM interacts with mRFP-‘RBD’5. We transiently transfected HEK293T cells with a mixture of two plasmids: one that produces eGFP-SSM and a second that produces mRFP-SSM-‘RBD’5, mRFP-‘RBD’5 or, as a negative control, mRFP (Fig. 4a). We then generated cell lysates and analyzed them in the presence of RNase A before and after immunoprecipitation using anti-GFP or mlgG.

Each mRFP-tagged protein and mRFP alone were expressed at comparable levels (Fig. 4b), and anti-GFP immunoprecipitated comparable amounts of eGFP-SSM (Fig. 4b). Although eGFP-SSM
The image includes a series of diagrams and figures (a, b, c, d) that illustrate the interaction between RBD5 and SSM proteins. The text discusses experiments involving these proteins, including co-immunoprecipitation and Western blotting, to study their interactions and functions. The results suggest that disrupting hSTAU1 dimerization inhibits UPF1 binding and SMD. The figures show cellular mRNA levels and protein expression levels before and after treatment with plasmids expressing RBD5 or a negative control. The text concludes with a discussion of the implications of these findings for the regulation of SMD in cells.
bind hUPF1 (Fig. 4b), indicates that hUPF1 binds hSTAU1 dimers more efficiently than hSTAU1 monomers.

We additionally examined the effect of mRFP-‘RBD’5 or eGFP-SSM, which we predicted would also inhibit hSTAU1 dimerization, on the efficiency of SMD by assaying the HEK293T SMD targets SOWAHC (formerly called FLI21870 in refs. 7, 10) mRNA, GAP43 mRNA and c-JUN (JUN) mRNA7,9. Each tagged protein was expressed in HEK293T cells comparably to its corresponding tag-only control (Fig. 4d). Transfections using plasmids expressing eGFP-SSM or mRFP-‘RBD’5 increased the abundance of each SMD target 2- to 2.5-fold relative to transfections using an empty vector (pcI-neo) or a plasmid expressing eGFP or mRFP, respectively, none of which affected SMD target abundance (Fig. 4d and Supplementary Fig. 4d). Thus, hSTAU1 dimerization is crucial for efficient SMD because dimerization augments hSTAU1 binding to hUPF1.

To define the minimal segment necessary for hSTAU1 dimerization in vivo, we transiently transfected HEK293T cells with pcI-neo–hSTAU155(R)-Flag and one of three siRNA-resistant plasmids that produce hSTAU155(R)-WT-Flag, hSTAU155(R)(C-term)-Flag (with deletion of residues C-terminal to the first α-helix of ‘RBD’5) or hSTAU155(R)Δ(SSM–‘RBD’5)-Flag (with deletion of the entire SSM–‘RBD’5 region), hereafter called WT, Δ(C-term) and Δ(SSM–‘RBD’5), respectively (Fig. 5a). We generated cell lysates and analyzed them in the presence of RNase A before and after immunoprecipitation using anti-Flag or, as a negative control, mIgG, or anti-HA or, as a negative control, rat IgG (rIgG).

The three Flag-tagged proteins were expressed at comparable levels before immunoprecipitation relative to each other (Fig. 5b) and relative to cellular hSTAU155 (Supplementary Fig. 5a) and were immunoprecipitated with comparable efficiencies using anti-Flag (Fig. 5b). The level at which hSTAU155-HA3 or cellular hUPF1 coimmunoprecipitated with Δ(SSM–‘RBD’5) was only ~10% of the level at which hSTAU155-HA3 or cellular hUPF1 coimmunoprecipitated with either WT or Δ(C-term) (Fig. 5b). Immunoprecipitations of the same transfections
using either anti-HA or, as a negative control, rIgG revealed that the level at which ∆(SSM-'RBD'5) coimmunoprecipitated with hSTAU155-HA3 was only ~10% the level at which WT or ∆(C-term) coimmunoprecipitated with hSTAU155-HA3 (Supplementary Fig. 5b). Thus, domain swapping between SSM and 'RBD'5 is the major determinant of hSTAU1 dimerization and can be achieved even when one of the interacting proteins lacks residues C-terminal to 'RBD'5 α1. Consistent with this conclusion, assays of the three detectable cellular hSTAU2 isoforms demonstrated that hSTAU2 coimmunoprecipitated with each hSTAU155(R)-Flag variant with the same relative efficiency as did hSTAU155-HA3 (Fig. 5b). Thus, hSTAU1 can homodimerize or heterodimerize with hSTAU2.

**Figure 6** hSTAU155 point mutations that disrupt dimerization inhibit hUPF1 binding and SMD, thereby precluding contributions of SMD toward inhibiting cell motility. (a–c) Western blotting (a,b) performed essentially as in Figure 5b,c except using the specified siRNA-resistant hSTAU155(R)-Flag expression plasmids. For c, which was performed essentially as in Figure 5d, see Supplementary Figure 6e for the RT-PCR analyses. IP, immunoprecipitation. Results are representative of three independently performed experiments. Error bars, s.e.m. *P < 0.05 determined using one-tailed t-test. (d,e) Western blotting of lysates before scrape injury (d) and phase-contrast microscopy at 0 and 16 h after scrape injury (e) of HaCaT keratinocytes (5 × 10⁶ cells per 100-mm dish) transiently transfected with the specified siRNA (100 nM) and, 1 d later, with the specified plasmid (3 µg). Scale bars, 100 µm.
Using anti-Flag to immunoprecipitate a hSTAU1<sup>SSM-(R)</sup>-Flag variant or anti-HA to immunoprecipitate hSTAU1<sup>SSM-HA</sup>, the coimmunoprecipitation of hUPF1 correlated with homodimerization ability (Fig. 5b and Supplementary Fig. 5b), which is in agreement with data obtained using mRFP-‘RBD’5 to disrupt dimerization (Fig. 4c). However, homodimerization did not augments the binding of hSTAU1<sup>SSM-HA</sup> to an SBS because SOWAHC mRNA and c-JUN mRNA each coimmunoprecipitated with WT, Δ(C-term) and Δ(SSM-‘RBD’5) to the same extent (Supplementary Fig. 5c).

As Δ(SSM-‘RBD’5) has residual dimerization activity (10% that of WT), and in view of reports that hSTAU1 ‘RBD’2 residues 37–79 interact with full-length hSTAU1<sup>SSM</sup> (ref. 25), we assayed the ability of E. coli–produced hSTAU1-‘RBD’2-RBD3 (residues 43–173) to dimerize. Gel filtration demonstrated that hSTAU1-‘RBD’2-RBD3 indeed migrates at the position expected of an ‘RBD’2-RBD3–‘RBD’2-RBD3 dimer (Supplementary Fig. 5d). The low level of residual activity suggests that the contribution of ‘RBD’2 to hSTAU1 dimerization is relatively minor and, as such, we did not pursue it further.

Inhibiting hSTAU1 dimerization should inhibit SMD on the basis of our finding that dimerization promotes the association of hSTAU1 with hUPF1. To test this hypothesis, we transiently transfected HEK293T cells with: (i) hSTAU1(A) siRNA<sup>8</sup>; (ii) a plasmid expressing one of the three hSTAU1<sup>SSM-(R)</sup>-Flag variants or, as a control, no protein; (iii) three plasmids that produce a firefly luciferase (FLUC) reporter mRNA, namely, FLUC-No SBS mRNA<sup>6</sup>, which lacks an SBS, FLUC-hARF1 SBS mRNA<sup>6</sup>, which contains the hARF1 SBS, and FLUC-hSERPINE1 3’ UTR mRNA<sup>8</sup>, which contains the hSERPINE1 SBS; and (iv) a reference plasmid that produces Renilla luciferase (RLUC) mRNA. In parallel, we transfected cells with (i) control siRNA<sup>8</sup>, (ii) a plasmid producing no hSTAU1<sup>SSM-(R)</sup>-Flag protein, (iii) the three FLUC reporter plasmids and (iv) the RLUC reference plasmid.

hSTAU1(A) siRNA reduced the abundance of cellular hSTAU1 to <~10% of that in control siRNA–treated cells, and each hSTAU1<sup>SSM-(R)</sup> Flag variant was expressed at a comparable abundance that approximated that of cellular hSTAU1<sup>SSM-HA</sup>–Flag (Fig. 5c). After normalizing the level of each FLUC mRNA to the level of RLUC mRNA, we found the normalized level of FLUC-No SBS mRNA, which is not an SMD target, to be essentially identical in all transfections (Fig. 5d and Supplementary Fig. 5e). In contrast, the normalized levels of FLUC-hARF1 SBS mRNA and FLUC-hSERPINE1 3’ UTR mRNA were increased approximately two-fold in the presence of hSTAU1(A) siRNA alone, as were the normalized levels of SOWAHC, GAP43 and c-JUN mRNAs, consistent with an inhibition of SMD (Fig. 5d). This inhibition was reversed by 50% when WT or Δ(C-term) was expressed but not when Δ(SSM-‘RBD’5) was expressed (Fig. 5d). Thus, WT and Δ(C-term) can functionally compensate for the siRNA-mediated downregulation of cellular hSTAU1 more efficiently than can Δ(SSM-‘RBD’5). These data indicate that hSTAU1 dimerization is important for SMD.

To define specific amino acids of hSTAU1 that contribute to domain swapping, we used our X-ray crystal structure to design seven variants of hSTAU1<sup>SSM-HA</sup>–Flag that, relative to the deletion-bearing variants, would harbor more subtle changes (Fig. 5a and Supplementary Fig. 6a). We designed mutations to target the SSM–‘RBD’5 interface and minimize any effects on the overlapping intramolecular hydrophobic interactions within ‘RBD’5 itself. When subjected to secondary structure predictions using PsiPred<sup>30,31</sup>, none of the mutations was predicted to disrupt the α-helical structure within which each resides.

Of the seven variants, only hSTAU1<sup>SSM-(R)</sup>-Flag harboring A375E, R376A, I472S and S473E (called hereafter Mut #7) disrupted hSTAU1<sup>SSM-(R)</sup>-Flag dimerization with hSTAU1<sup>SSM-HA</sup> (Supplementary Fig. 6b). This variant contains a bulky substitution at residue 375, a change at residue 376 that disrupts one of the two polar interactions in the hSTAU1 SSM–‘RBD’5 interface and L472S and S473E, both of which target residues within ‘RBD’5 α2 that interact with SSM α1 (Fig. 1c,d). Notably, T371R and Q419A, which disrupt the second polar interaction in the hSTAU1 SSM–‘RBD’5 interface, do not affect dimerization either individually or when combined in cis (Supplementary Fig. 6b).

Western blotting of lysates of HEK293T cells that transiently expressed comparable amounts of Mut #7 and hSTAU1<sup>SSM-HA</sup> (Fig. 6a and Supplementary Fig. 6c) at levels that approximated that of cellular hSTAU1<sup>SSM</sup> (Supplementary Fig. 6b) revealed that hSTAU1<sup>SSM-HA</sup> cellular hUPF1 and isoforms of cellular hSTAU2 did not coimmunoprecipitate efficiently with Mut #7 (Fig. 6a and Supplementary Fig. 6c). Also, the binding of Mut #7 to the SMD targets SOWAHC and c-JUN mRNAs was not compromised (Supplementary Fig. 6d). Consistent with the importance of hSTAU1 dimerization to SMD, Mut #7 was less able to reverse the hSTAU1(A) siRNA–mediated inhibition of SMD than was WT (Fig. 6b,c).

**DISRUPTING STAU1 DIMERIZATION INHIBITS WOUND HEALING**

Downregulating the levels of SERPINE1 and RAB11FIP1 mRNAs, which are SMD targets, increases keratinocyte motility in a scrape injury–repair (wound-healing) assay<sup>10</sup>. To test the physiological importance of disrupting hSTAU1 dimerization, we expressed WT, Δ(C-term), Δ(SSM-‘RBD’5) and Mut #7 individually at equal levels in human HaCaT keratinocytes that had been treated with hSTAU1(A) siRNA, which reduced the cellular hSTAU1 abundance to 10% of that in control siRNA–treated cells (Fig. 6d, pcle-no served as a control). After 16 h, enhanced keratinocyte motility was evident in the presence of hSTAU1 siRNA alone, consistent with SERPINE1 and RAB11FIP1 proteins enhancing wound healing<sup>10</sup>, and also when cellular hSTAU1 was replaced by Δ(SSM-‘RBD’5) or Mut #7, neither of which can dimerize to mediate SMD (Fig. 6e). On the basis of these findings and data showing that replacing cellular hSTAU1 with either WT or Δ(C-term), each of which supports hSTAU1 dimerization, had no effect on keratinocyte motility (Fig. 6e), we conclude that contributions of hSTAU1 dimerization to the efficiency of SMD are indeed important in promoting wound healing.

**DISCUSSION**

hSTAU1 homodimerization requires a previously unknown motif

Here we describe the hSTAU1 SSM, which is a two-helix motif (Fig. 1) that interacts with dsRNA binding–deficient ‘RBD’5 of another hSTAU1 molecule (Figs. 1, 3–6 and Supplementary Figs. 2 and 4–6). We propose that the SSM is a modular adaptation in many, and possibly all, vertebrate STAU homologs that mediates STAU dimerization through its interaction with ‘RBD’5. Although the connectivity between the SSM and ‘RBD’5 cannot be modeled, we suggest that the dynamic nature of the linker (Supplementary Fig. 2c) allows hSTAU1 SSM–‘RBD’5 to exist in both monomeric and dimeric states, and both states potentially exist in the crystal structure. We support our crystallographic model for dimerization by demonstrating that hSTAU1 SSM–‘RBD’5 dimers form in solution in vitro (Fig. 3) and in cells (Figs. 4–6 and Supplementary Figs. 4–6). If hSTAU1 multimerization were to occur in cells, it would probably involve not only SSM interacting with ‘RBD’5 in trans (Fig. 4) but also weaker contributions from ‘RBD’2 (ref. 25 and Supplementary Fig. 5). Possibly, dimerization through intermolecular ‘RBD’2–‘RBD’2 interactions would promote trans over cis interactions between SSM and ‘RBD’5.

Data indicate that the minimal region of ‘RBD’5 from one molecule that is needed to interact with the SSM from another is ‘RBD’5 α1.
First, sequences that reside C-terminal to ‘RBD’5 α1 are not required for hSTAU1-hSTAU1 dimerization (Fig. 5). Second, the smallest hSTAU2 isoform coimmunoprecipitates with hSTAU1 even though its ‘RBD’5 consists of only α1 and L1 (Fig. 5 and Supplementary Fig. 1). Thus, all STAU1 isoforms can dimerize, if not multimerize, with themselves and/or all STAU2 isoforms. We suggest that ‘RBD’5 α2 may stabilize dimer formation given that the SSM-‘RBD’5 interaction can be disrupted by simultaneously mutating both SSM and ‘RBD’5 α2 (Fig. 6). Additionally, mutations at the SSM-‘RBD’5 α1 interface do not effectively disrupt dimerization, possibly because of the compensating presence of ‘RBD’5 α2 (Supplementary Fig. 6).

hSTAU1 homodimerization contributes to SMD

Compared to hSTAU1 monomers, hSTAU1 dimers bind hUPF1 more efficiently (and mediate SMD more effectively) without promoting dsRNA binding (Figs. 4–6 and Supplementary Figs. 4–6). Thus, cells may regulate SMD by controlling hSTAU1 abundance32 and, therefore, dimer formation (Fig. 7). There is clear evidence that multiple hSTAU155 molecules can bind a single dsRNA. For example, multiple hSTAU155 molecules bind the hARF1 SMD target in cells25 and mRNAs containing as many as 250 CUG repeats that typify patients with myotonic dystrophy in vitro33. Also, our finding that hSTAU155 stabilizes the relatively large (86–298 imperfectly base-paired) regions that constitute intermolecular SBSs formed between an mRNA and a long noncoding RNA through Alu-element base pairing34 within an mRNA 3′UTR of an mR NA that is targeted for SMD and promote its decay. hSTAU1 can bind to an mRNA and a long noncoding RNA through Alu-element base pairing34 within an mRNA 3′UTR. Dimerization is mediated by reciprocal domain-swapped interactions between the SSM of one hSTAU1 molecule (blue) and the ‘RBD’5 of another (green). Note that ‘RBD’2 may also facilitate hSTAU1-hSTAU1 dimerization25, which could change the orientation of the hSTAU1 dimer from what is shown here. hSTAU1 dimerization promotes hUPF1 binding. Binding activates UPF1 helicase activity and initiates mRNA decay40. 7mG, 7-methyl guanosine cap; AUG, translation initiation codon; Ter, normal termination codon; Aκ, poly(A) tail. For emphasis, molecules are not shown to scale.

The RBD fold as a template for functional diversity

As reported here, the combination of a modified RBD (hSTAU1 ‘RBD’5) within the context of an adaptor region (hSTAU1 SSM) can promote greater functionality within the larger, often modular and flexible framework of RBD-containing proteins. In support of this view, modifications that consist of an L1 cysteine and an L3 histidine within the RBD of the Schizosaccharomyces pombe Dicer DCR1 protein work together with a 33-residue region that resides C-terminal to the RBD to form a zinc-coordination motif that is required for nuclear retention and, possibly, dsDNA binding38.

‘RBDs that do not bind dsRNA may also acquire new functions independently of adjacent regions. For example, ‘RBD’5 of D. melanogaster STAU has adapted to bind the Miranda protein that is required for proper localization of prospero mRNA39,40. In addition, human TAR RNA-binding protein 2 contains three RBDs, the most C-terminal of which binds Dicer instead of dsRNA41,42. Furthermore, ‘RBD’3 of Xenopus laevis RNA-binding protein A, similarly to its human homolog p53-associated cellular protein, seems to homodimerize independent of an accessory region43. It will be interesting to determine whether hSTAU1 ‘RBD’2-mediated dimerization25 involves an adaptor motif or occurs solely through the RBD fold.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The hSTAU1 SSM-‘RBD’5’s coordinates and structure factors have been deposited in the PDB with accession code 4DKK.

Note: Supplementary information is available in the online version of the paper.

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ONLINE METHODS

Sequence alignments. Sequences were obtained from NCBI. Multiple protein sequence alignments were performed using ClustalW2 (v.1.4) within BioEdit, which was used to generate figures. To generate Supplementary Figure 1a, STAU protein sequences from the following vertebrate classes were used for the alignment: fish (zebrafish, D. rerio, NP_991124.1), amphibians (African clawed frog, Xenopus laevis, NP_001085239.1 for STAU-1 and NP_001089618.1 for STAU-2), reptiles (Carolina anole, Anolis carolinensis, XP_003226868.1), birds (zebra finch, Taeniopygia guttata, XP_002188609.1) and mammals (human, Homo sapiens, NP_004593.2 for STAU-1, NP_001157856.1 for STAU2 and NP_001157853.1 for STAU2; and mouse, Mus musculus, NP_001103375.1 for STAU-1 and NP_001104742.1 for STAU-2).

Plasmid constructions. See Supplementary Note 2.

Protein expression in E. coli and protein purification. E. coli BL21(DE3) transformed with pGEX-6p-1-hSTAU1-SSM-‘RBD’5 was propagated in multiple l-l cultures of Luria Broth supplemented with ampicillin (100 mg l\(^{-1}\)) to an optical density at 600 nm (\(OD_{600}\)) of ~0.5, at which time 300 µl of 1 M isopropyl \(\beta\)-D-thiogalactopyranoside was added to each liter, and the temperature was reduced from 37°C to 30°C. The next morning, cells were collected at ~7,000g and 4°C and either used directly or flash frozen in liquid nitrogen for storage at ~80°C.

Cell pellets were resuspended in ~40 ml of Buffer A (1 M NaCl and 25 mM Tris-HCl, pH 8) to which was added 50 µl of 0.93 M DTT, 500 µl of 100 mM PMSE, 50 µl of 0.5 M EDTA, pH 8, 500 µl of 80 mg ml\(^{-1}\) lysozyme and a proteinase inhibitor tablet (Roche). Cells were lysed using sonication, and lysates were cleared by centrifugation at 17,000g for 30 min at 4°C. The soluble portion was removed and loaded on a GSTrap HP column (GE Healthcare), washed with 1 M NaCl, 25 mM HEPES, pH 8 (which was sometimes replaced with Buffer A), washed with gel-filtration buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, and 1.3 mM DTT; this step was sometimes omitted) and then eluted with 0.3 g of glutathione (reduced, free acid) dissolved in 100 ml of gel-filtration buffer. A ~1-mg aliquot of PreScission Protease (GE Healthcare) was added to ~50 ml of the dissolved protein association model using the Lamm equation.

Sequence alignments were performed using Clustal W2 (v.1.4) within BioEdit, for STAU259; and mouse, Mus musculus, NP_001085239.1 for STAU-1, NP_001130375.1 for STAU-2 and NP_001104742.1 for STAU-2.

Protein crystallization and structure determinations. Native crystals were produced from gel-filtration–purified hSTAU1 SSM–‘RBD’5 using either the sitting-drop method (native 1 crystal) or the hanging-drop method (native 2 crystal) (Table 1). The native 1 crystal was collected using the CHESS beamline F1 under a cryostream at a wavelength of 0.9177 Å (Table 1). The native 2 crystal was collected remotely at the SSRL beamline 9-2 under a cryostream at a wavelength of 0.9793 Å (Table 1). An initial model was built using low-resolution SAD phases (0.432 figure of merit) from data collected in house on an ethyl mercuric phosphate-soaked crystal (Ethyl-Hg-SAD) under a cryostream at a wavelength of 1.5418 Å (Table 1). Model coordinates were used for molecular replacement and refined against the 2.2-Å native 1 data set (Table 1), and the resulting coordinates were subsequently refined against the 1.7-Å native 2 data set. For the final structure, MolProbity reported a clash score of 19.14 and that 97% of the residues were in the favored region of the Ramachandran plot with no outliers. Structure figures were generated using PyMOL (Schrodinger, LLC). See Supplementary Note 3 for crystallization and structure determination details.

Western blotting, RT-PCR and immunoprecipitations. Protein was electrophoresed in SDS-polyacrylamide, transferred to Hybond ECL nitrocellulose (Amersham) and probed with antibodies that recognize Flag (Sigma, F315, 1:5,000), HA (Roche, 11867423001, 1:1,000), calnexin (StressGen, SPA-860, 1:1,000), UPF1 (ref. 7, 1:1,000), STAU1 (a gift from the Ortín lab, 1:1,000), STAU2 (Sigma, HPA019155, 1:500). Immunoreactivity was assessed using SuperSignal West Pico (Pierce Biotechnology). After autoradiography, films were quantitated using ImageQuant (Molecular Dynamics).

Reverse transcription and PCR amplification were performed as previously described. RT-PCR products were electrophoresed in 5% polyacrylamide and quantified by PhosphorImaging (Molecular Dynamics). The five leftmost lanes of each figure represent two-fold serial dilutions of RNA. A standard curve was derived from these five lanes and used to calculate the relative abundance of each mRNA from different transfections. P values were determined using one-tailed t-test.

Immunoprecipitations were performed using anti-GFP (Abcam), anti-HA (Roche) or anti-Flag (Sigma). To determine immunoprecipitation and coimmunoprecipitation efficiencies. ImageQuant values that were obtained by western blotting samples before or after immunoprecipitation were superimposed on the values obtained for the three-fold serial dilutions of protein before...
immunoprecipitation that are provided in the four leftmost lanes of each western blot. For each protein, the value after immunoprecipitation was normalized to the value before immunoprecipitation, and the values were then compared. See Supplementary Table 2 for the immunoprecipitation and coimmunoprecipitation efficiencies for each experiment.

Wound-healing assays. The methods used were as described10. Cells were imaged with a Nikon Eclipse TE2000-U inverted fluorescence microscope.

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