The NMR and X-ray Structures of the \textit{Saccharomyces cerevisiae} Vts1 SAM Domain Define a Surface for the Recognition of RNA Hairpins

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The SAM domain of the \textit{Saccharomyces cerevisiae} post-transcriptional regulator Vts1 has a high affinity towards RNA hairpins containing a CUGGC pentaloop. We present the 1.6 Å X-ray crystal structure of the Vts1 SAM domain in its unliganded state, and the NMR solution structure of this domain in its RNA-bound state. Both structures reveal a canonical five helix SAM domain flanked by additional secondary structural elements at the N and C termini. The two structures are essentially identical, implying that no major structural rearrangements occur upon RNA binding. Amide chemical shift changes map the RNA-binding site to a shallow, basic patch at the junction of helix a5 and the loop connecting helices a1 and a2.

The SAM domain of \textit{Saccharomyces cerevisiae} Vts1, the object of this study, binds RNA \textit{in vitro} with the same sequence specificity and affinity as Smaug.\textsuperscript{7} Vts1 promotes the degradation of a reporter gene containing three consecutive SRE sequences. However, unlike Smaug, which acts upon \textit{nanos} gene transcripts, no endogenous targets of Vts1 have been identified. Though precise functional roles for Vts1 remain undefined, Vts1 has been implicated in the general processes of vesicular transport\textsuperscript{9} and sporulation.\textsuperscript{10} The Smaug and Vts1 SAM domains bind RNA hairpins bearing either a 5′-CUGGC-3′ pentaloop or a 5′-CUGG-3′ tetraloop with great affinity.\textsuperscript{7} Substitutions of Cyt1, Gua3 and Gua4 (bases are numbered according to their position in the pentaloop) with any other base reduces binding by more than two orders of magnitude, while substitutions of Ura2 or Cyt5 have no effect.\textsuperscript{7}

Towards understanding the structural aspects of RNA recognition by Vts1, the crystal structure of its unliganded SAM domain and the NMR structure of the SAM domain in its RNA-bound state were determined. Amide chemical shift differences

Abbreviations used: SAM, sterile alpha motif; SRE, Smaug recognition element.
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between the free and bound states identify a basic patch that corroborates previous mutagenesis studies. Superposition of the free and bound protein structures indicates that SRE binding does not induce global or local conformational changes in the Vts1 SAM domain.\textsuperscript{10}

**Delineating the boundaries of the Vts1 SAM domain**

Initially, we expressed a large fragment of Vts1 (residues 407–523) that encompassed the canonical SAM domain (residues 448–512) and a sizable N-terminal region fused to glutathione-S-transferase. Although Vts1 does not possess a C-terminal pseudo HEAT repeat analogous topology domain like its counterpart Smaug, deletion analysis indicated that sequences flanking the canonical SAM domain sequence were important for protein folding and solubility. The core RNA-binding fragment of Vts1 was further delimited by treatment with the protease thermolysin. The boundaries of a protease-resistant fragment (residues 436–523) were deduced by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and tandem mass spectrometry sequencing of trypsinized peptides. The thermolysin-resistant fragment was functionally indistinguishable from the larger Vts1\textsuperscript{407–523} fragment on the basis of SRE RNA binding affinity (\(K_d \approx 30\) nM) and specificity (Figure 1(a)).

**The crystal structure of the unliganded Vts1 SAM domain**

Crystals of the thermolysin-resistant fragment of Vts1 were obtained using a commercially available sparse matrix screen. Phase determination was performed by single isomorphous replacement with anomalous scattering on a mercury derivative of an

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**Figure 1.** The crystal structure of free Vts1 SAM and the NMR structure of SRE-bound Vts1 SAM are essentially identical. A gene fragment encompassing the SAM domain (residues 407–523) of *S. cerevisiae* VTS1 was amplified by PCR methods and inserted into a modified pGEX vector. The resulting glutathione-S-transferase fusion protein was purified by affinity chromatography from soluble extracts of *E. coli* BL21(DE3) grown in a BioFlow 110 fermentor at 25 °C. Isotopically labeled Vts1\textsuperscript{407–526} was obtained from fermentations in minimal M9 medium containing 1.0 g/l \([U-98\%] [15N]\) ammonium chloride and 4.0 g/l \([U-99\%] [13C]\) glucose as the sole sources of nitrogen and carbon, respectively. Vts1\textsuperscript{407–523} was cleaved from the fusion protein with TEV protease. Proteins were further purified and buffer-exchanged using S-100 gel-filtration chromatography. For crystallography, the 13 kDa Vts1 fragment was digested with thermolysin to produce a minimal 10 kDa fragment, Vts1\textsuperscript{436–523}, that was purified further by heparin chromatography. A 19 nt SRE RNA (5'-GGAGGCUCCUGGCAGUCUUC-3') was prepared in milligram quantities for NMR spectroscopy by phage T7 RNA polymerase-driven, in vitro transcription.\textsuperscript{17} The SRE RNA was purified by denaturing 20% PAGE and electrophoresed. Renaturation of the SRE RNA hairpin was achieved by rapidly chilling a solution that was preheated to 94 °C. (a) A fluorescence polarization binding assay of the thermolysin-resistant Vts1 SAM domain demonstrates high-affinity binding for the SRE (5'-AGGCUCCUGGCAGUCUUC-3') but not a mutant SRE bearing substitutions at the first and third loop positions. (b) A modest decrease in binding affinity is observed when either 5 mM EDTA or 10 mM Ca\textsuperscript{2+} is present in the binding buffer. (c) Superposition of the free Vts1 SAM domain crystal structure with the bound NMR structure reveals no significant conformational changes. The SRE binding site is indicated in red. Superposition of the crystal structures of the Vts1 SAM domain with the (d) EphA4 SAM domain and the (e) Smaug SAM domain. (f) Superposition of the 20 lowest energy NMR structures of the RNA-bound Vts1 SAM domain.
A single copy of Vts1436–523 and an anomalous scattering ion (possibly Ca\(^{2+}\)) is present in the asymmetric unit. Though a divalent metal ion (Mg\(^{2+}\), Ca\(^{2+}\), Sr\(^{2+}\), or Ba\(^{2+}\)) is required for crystallization, fluorescence polarization binding assays performed in the presence of calcium and chelating agents revealed that metal ions do not play a significant role in SRE binding (Figure 1(b)). The crystal structure at 1.6 Å resolution (Figure 1(c); Table 1) comprises residues 442–523 of Vts1, with six disordered N-terminal residues (436–441). The hallmark five-helix bundle (\(z_1\)–\(a_5\), residues 455–515) of the SAM domain is apparent and superimposes well with the crystal structure of the EphA4 SAM domain (RMSD 1.3 Å; Figure 1(d)) and, to a lesser degree, with the Smaug SAM domain (RMSD 2.2 Å; Figure 1(e)). Two short \(z\)-helices denoted \(z_0\), (residues 449–453) and \(\alpha(−1)\), (residues 443–447) and one \(3_{10}\) helix (residues 520–522) pack against the N terminus of helix \(z_1\) and the C-terminal portion of helix \(a_5\). In Smaug, these additional structural elements are donated by the pseudo HEAT repeat analogous topology domain.\(^5\) From sequence comparisons, helix \(z_0\) is conserved among all fungal homologs of Vts1, while helix \(\alpha(−1)\) and the \(3_{10}\) helix are conserved only among a sub-group of closely related yeast species.

### Table 1. Data collection, phasing and refinement statistics of the free Vts1 SAM domain crystal structure

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<th>A472C-HgCl</th>
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<td>Bond angles (deg.)</td>
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A stock solution of Vts1 was prepared in 20 mM Hepes (pH 7.0), 100 mM NaCl. Crystals were grown from microseeded drops of 3 mM Vts1 mixed in equal parts with a reservoir solution containing 50 mM Tris–HCl (pH 8.5), 30% (v/v) PEG 4000, 200 mM NaH\(_2\)PO\(_4\), 10 mM CaCl\(_2\). In 24–48 h, crystals of 0.1 mm containing 50 mM Tris–HCl (pH 8.5), 30% (w/v) PEG 4000, 0.02% (w/v) NaN\(_3\), and 10% 2H\(_2\)O. Spectra were acquired at 20 °C on a Bruker Avance 600 MHz spectrometer and a Varian Inova 800 MHz spectrometer (NANUC, Edmonton, AB). A Vts1-RNA complex was prepared by titrating protein into an RNA solution and following changes in RNA imino resonances at 5 °C. Backbone assignments of free and bound Vts1 were obtained from HNCA, CBCA(CO)NH and HCCH-TOCSY spectra. NOE crosspeaks from \(^{1}H-^{15}N-HSQC-NOESY\) and \(^{13}C-^{15}N-HSQC-NOESY\) spectra (100 ms mixing time) were calibrated from 1.8–5.0 Å using TALOS.\(^{24}\) Initial structures were calculated with CYANA.\(^{26}\) The best 100 structures according to lowest energy were subjected to one round of water refinement.\(^{27}\) Analysis of the 20 best water-refined structures using PROCHECK-NMR\(^{28}\) indicates that 92.5% of the amino acid residues are located in the most favored region of the Ramachandran map, with 7.3% in additionally allowed regions and 0.1% in generously allowed regions.
The NMR structure of the Vts1 SAM domain in the RNA-bound state

Many RNA-binding domains place a secondary structure element into the RNA major groove or present a cleft for RNA docking. These structural features are absent from the unliganded Vts1 SAM domain, as revealed by X-ray crystallography. To determine if RNA-binding induces structural changes in the Vts1 SAM domain which, in turn, create a deep binding pocket or protrusion, we determined the solution structure of Vts1407–523 bound to 19 nt SRE RNA pentaloop hairpin at a stoichiometric ratio of 1:1. The overall backbone RMSD of the 20 lowest energy conformers is 0.77 Å based on 1729 NOE-derived distance restraints and 73 database-derived torsion angle restraints (Figure 1(f); Table 2). The resulting structure of the Vts1 SAM domain in its bound state is essentially identical with the X-ray structure of the Vts1 SAM domain in its free state. The backbone (N, Cα, Cβ) atoms of the free X-ray structure and the bound NMR structure of Vts1 SAM domains superimpose with an RMSD of 1.26 Å, comparable to the precision of the NMR structure determination (Figure 1(c)). This high degree of structural similarity leads us to conclude that SRE binding does not induce a major conformational rearrangement of the Vts1 SAM domain.

Mapping the SRE RNA-binding site

RNA binding induces a number of 1H, 15N chemical shift changes in the Vts1 SAM domain (Figure 2(a)). The majority of these changes localize to a conserved positively charged surface patch defined by the z1-z2 loop and the N terminus of helix z5 (Figure 2(b)). Of the six amide resonances (Leu465, His466, Leu496, Gly497, Ala498, and Lys501) that shift significantly upon RNA binding, all but Leu496 are absolutely conserved among Vts1 homologs, including Drosophila Smaug.7,8 Significant chemical shift changes were observed also for the guanidino N3/H3 resonances of Arg464 and Arg500 (data not shown), suggesting that these charged groups contact RNA. Among Vts1 homologs, Arg464 is absolutely conserved, while Arg500 may be substituted with other polar amino acid residues. Overall, the binding site deduced by chemical shift mapping corroborates previous mutagenesis studies on Vts1 and Smaug.7,8

Among 11 Cα atoms in the deduced RNA binding site (residues 464–467, 496–502), the free and bound states superimpose to 0.62 Å RMSD (Figure 1(c)).

Figure 2. The RNA-binding surface on the Vts1 SAM domain. (a) Weighted-averaged18 amide 1H and 15N chemical shift changes upon SRE RNA binding where \( \delta_{\text{av}} \) = \( \delta_{\text{H}} + (8N/5)^{1/2} \delta_{\text{N}} \). Bars are colored according to the magnitude of the chemical shift change (red, > 0.3 ppm; yellow, > 0.1 ppm). (b) Chemical shift changes, sequence conservation, and electrostatic charge are mapped onto the surface of the free X-ray structure of Vts1 SAM domain. HSQC perturbations are colored according to (a). The linewidth of Lys467 sharpens significantly upon RNA binding and is colored orange. Sequence identity (in purple) and sequence similarity (in pink) among Vts1 homologs coincides with the SRE binding surface deduced from chemical shift perturbations. The RNA-binding site demonstrates basic charge distribution (red; negative potential; blue, positive potential). Molecular structure representations and electrostatic calculations were made with pyMOL (http://pymol.sourceforge.net).
This indicates that the RNA-binding site on the SAM domain exists in a preconfigured, competent state for SRE binding. Moreover, the shallow depth and limited size of the binding site suggests that the SAM domain may discriminate only a portion of the SRE.

SAM domain-mediated protein–protein interactions typically occur through two surfaces termed mid-loop (ML) and end-helix (EH), as observed in the structures of the Tel SAM domain homo-oligomer,14 the Yan/Mae SAM domain heterodimer,15 and the Ph/Scm SAM domain heterodimer.16 The RNA-binding surface does not overlap the corresponding ML surface and partially overlap the corresponding EH surfaces of the Vts1 SAM domain. If Vts1 SAM engages other proteins through its EH or ML surfaces, these protein–protein interactions may regulate Vts1 function either by obscuring SRE binding or by recruiting additional factors necessary for transcript regulation.

**Protein Data Bank accession codes**

Coordinates of Vts1 SAM in its unbound and SRE-bound forms have been deposited in the Protein Data Bank with accession identifiers 2D3D and 2B6G, respectively.

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**References**


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