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SARAF Luminal Domain Structure Reveals a Novel Domain-Swapped β-Sandwich Fold Important for SOCE Modulation

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Abstract

Store-Operated Calcium Entry (SOCE) plays key roles in cell proliferation, muscle contraction, immune responses, and memory formation. The coordinated interactions of a number of proteins from the plasma and endoplasmic reticulum membranes control SOCE to replenish internal Ca²⁺ stores and generate intracellular Ca²⁺ signals. SARAF, an endoplasmic reticulum resident component of the SOCE pathway having no homology to any characterized protein, serves as an important brake on SOCE. Here, we describe the X-ray crystal structure of the SARAF luminal domain, SARAF_L. This domain forms a novel 10-stranded β -sandwich fold that includes a set of three conserved disulfide bonds, denoted the "SARAF-fold." The structure reveals a domain-swapped dimer in which the last two β -strands (β 9 and β 10) are exchanged forming a region denoted the "SARAF luminal switch" that is essential for dimerization. Sequence comparisons reveal that the SARAF-fold is highly conserved in vertebrates and in a variety of pathologic fungi. Förster resonance energy transfer experiments using full-length SARAF validate the formation of the domain-swapped dimer in cells and demonstrate that dimerization is reversible. A designed variant lacking the SARAF luminal switch shows that the domain swapping is essential to function and indicates that the SARAF dimer accelerates SOCE inactivation.

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Calcium is a potent second messenger required for diverse cellular signaling processes that occur over a wide range of timescales such as vesicle release (μ s), fertilization (minutes), and proliferation and apoptosis (hours) [1,2]. Consequently, cells use a multitude of systems to control cytoplasmic Ca²⁺ level changes. Signaling in both non-excitable and excitable cells is frequently initiated by stimulation of a G-protein coupled receptors and receptor tyrosine kinases [3,4] that trigger inositol triphosphate (IP₃)-mediated release of Ca²⁺ from intracellular stores. The resulting intracellular Ca²⁺ store depletion activates a process called Store-Operated Calcium Entry (SOCE) that works to replenish the internal Ca^{2+} stores and that affects a range of responses, such as proliferation, transcription, and cell motility [5–9].

The prototypical mediator of SOCE is the calcium release-activated calcium (CRAC) channel formed by the plasma membrane (PM) pore-forming subunit, Orai, and an endoplasmic reticulum (ER) Ca²⁺ sensor and channel activator, STIM [10–13]. Both SOCE components have multiple isoforms of which the best studied are STIM1 and Orai1. SOCE activation involves an elegant mechanism that results in the clustering of both STIM and Orai at

the ER–PM junctions [14–16]. Depletion of ER Ca²⁺ induces STIM1 oligomerization and translocation to the ER–PM junctions, where it binds to Orai1 and initiates Ca²⁺ influx [17–22]. Once SOCE is initiated, Ca²⁺ from CRAC channels initiates autoregulatory deactivation and inactivation processes that shape the duration and magnitude of the Ca²⁺ signal. Two types of Ca²⁺ dependent inactivation have been described: a fast process that occurs on the millisecond time scale, and a slow process that develops over multiple minutes [23]. The ER resident, single-pass transmembrane SARAF [24] (for *S*OCE-*A*ssociated *R*egul*A*tory *F*actor) is a central facilitator of the slow Ca²⁺ dependent inactivation of CRAC channels.

SARAF lacks homology to any known protein. Previous studies established that the SARAF elements on either side of the membrane encode two distinct functions. The SARAF cytosolic domain is required for driving SOCE inactivation through interaction with STIM [24-26], whereas the luminal domain regulates SOCE inactivation by responding to changes in ER Ca²⁺ levels [24]. Here, we present the X-ray crystal structure of the SARAF ER luminal domain, SARAF₁. This domain forms a domain-swapped dimer arrangement built from a novel B-sandwich fold that we term the "SARAF fold." Cross-linking, analytical ultracentrifugation, and Förster resonance energy transfer (FRET) experiments demonstrate that self-association of SARAF_L and full-length SARAF in cells depends on the domain-swapped element denoted as the "SARAF luminal switch." Finally, CRAC current recording in cells that express wildtype SARAF or dimerization-incompetent SARAF shows that self-association via the SARAF luminal switch is critical for control of CRAC currents.

Results

SARAF luminal domain dimerizes using a novel, conserved domain-swapped β-sandwich fold

The SARAF ER luminal domain contains six cysteines and bears no sequence homology to any protein of known structure (Fig. 1a and b). Therefore, we set out to define its structure to gain insight into how it might affect SOCE. Extensive screening identified a human SARAF luminal domain construct that could be expressed in *Escherichia coli* Shuffle Express cells, purified to homogeneity, and crystal-lized. This construct, denoted SARAF_L, excludes the N-terminal signal peptide, encompasses luminal domain residues 30–164, and ends eight residues before the putative transmembrane helix [24,27] (Fig. 1a). SARAF_L crystals diffracted X-rays to a resolution of 1.9 Å, and the structure was determined by single isomorphous replacement with anomalous

scattering (SIRAS) using a single platinum derivative $(K_2Pt(NO)_4)$ that diffracted X-rays to 2.15 Å (Fig. S1a, Table S1). SARAF_L crystallized with two molecules in the asymmetric unit. One copy had continuous electron density from N- to C-terminus, while the other showed short regions of disorder between residues 88–93 and 149–156 (Fig. S1b). Hence, the structural description focuses on the complete copy.

SARAF₁ forms a domain-swapped dimer comprising a 10 B-strand barrel having three conserved disulfides (Fig. 1b-d). There are two well-ordered extended loops that connect 62–64 and 66–68. Both are stabilized by the presence of a short intervening B-strand midway through the loop that forms B-sheet interactions with other β -strands (Fig. 1c and d). The β 3 strand in the middle of the β 2– β 4 loop makes a parallel β -sheet with β 8, whereas the β 7 strand in the $\beta 6-\beta 8$ loop forms an anti-parallel interaction with $\beta 1$. The $\beta 2-\beta 4$ and $\beta 6-\beta 8$ loops are further constrained by the Cys66-Cys73 and Cys114-Cys130 disulfides, respectively. The remaining disulfide, Cys83-Cys97, is buried in the core of the protein and links β 4 and β 5. There is a single α -helix, α 1, that follows $\beta 8$. This helix extends from the body of the structure and mediates a domain swap through which β -strands β 9 and β 10 complete the β -sheet fold of the other member of the dimer (Fig. 1e).

The domain-swapped β9–β10 element comprising GIn152–Lys164 makes extensive interactions with the SARAF₁ core that are mediated by the formation of anti-parallel β-sheet main chain hydrogen bonds between $\beta 9-\beta 5$ and a short parallel β -sheet made between $\beta 10$ and $\beta 6$ and that bury 2232 Å² per tailbody interface. (Fig. 1c–e). The backbone β -sheet interactions are accompanied by a number of sidechain-mediated interactions. Cross-strand hydrogen bonds are formed by His153-Tyr105 (Fig. S1c), a network comprising Ser157, Ser159, and Thr110 (Fig. S1d), and a Try163 hydroxyl and Tyr91 carbonyl interaction (Fig. 1e). Phe155 and Phe158 sidechains rest in shallow grooves on the surface the SARAF_L core, while the Tyr161 sidechain is buried in a largely hydrophobic pocket (Fig. 1e). Finally, the Lys164 C-terminal carboxylate forms a hydrogen bond to the amide of Gly116 and salt-bridge with the Arg57 sidechain (Fig. 1e). Despite being involved in a Ca²⁺-dependent process, SARAF lacks any obvious Ca²⁺-binding motifs [24]. Examination of the surface electrostatic potential (Fig. 2) revealed a few disperse regions of negative potential, but none that would indicate a site for Ca²⁺-binding, as well as a large positive patch in a pocket near SS3. In accordance with this lack of clear Ca2+-binding motifs, even though the structure was determined in the presence of 1 mM CaCl₂, we found no crystallographic evidence for Ca²⁺ binding to SARAF_L.

Searches for structural homology between SARAF_L and proteins of known structure using the DALI database [28] revealed no strong matches,

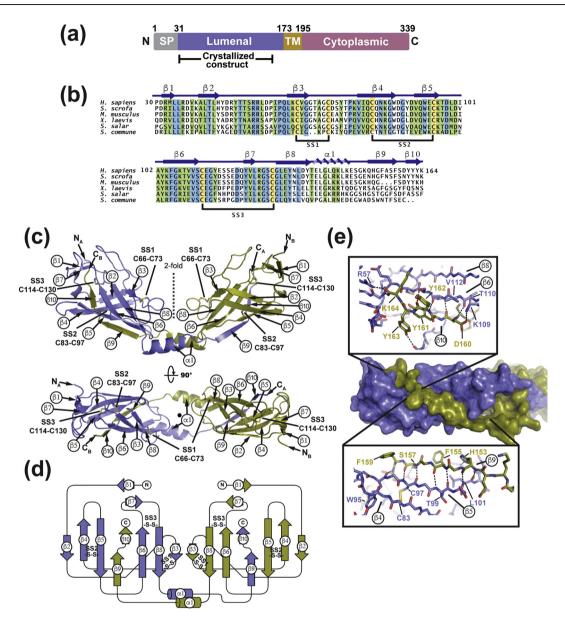


Fig. 1. Structure of SARAF_L. (a) SARAF schematic. Signal peptide (SP, gray), luminal (purple), transmembrane (TM, brown), and cytoplasmic (magenta) domains and amino acid boundaries of each are indicated. Extent of crystallized SARAF_L construct is shown. (b) SARAF_L sequence comparison from human (*H. sapiens*), pig (*S. scrofa*), mouse (*M. musculus*), frog (*X. laevis*), salmon (*S. salar*), and mushroom (*S. commune*). Invariant (blue), conserved (green) and cysteines (yellow) are highlighted. Secondary structure elements and disulfide bonds (SS1, SS2, and SS3) are shown. (c) Structure of the SARAF_L domain-swapped dimer. N and C termini (N_A, N_B, C_A, and C_B) and secondary structure elements of each chain are labeled. Disulfide bonds are shown as sticks and are labeled. Chains A and B are slate and deep olive, respectively. (d) SARAF_L domain-swapped dimer topology diagram. "S–S" denotes disulfide bonds. (e) Detailed view of interactions of domain-swapped β9 and β10 with the SARAF_L core. Dashed lines indicate hydrogen bonds.

indicating that SARAF_L has a unique fold. Relaxation of the similarity criteria to allow for a generous *Z*-score cutoff (>2) identified a set of β -sheet structures that include the γ -COPI appendage domain (1R4X), Xenavidin (2UYW), Avidin-related protein 2 (1WBI), and a conserved domain from *Bacillus anthracis* (3FBQ) (*Z*-scores of 2.3, 2.2, 2.1, and 2.0, respectively)(Fig. 3). However, strand connectivity analysis reveals that SARAF_L is substantially different from each of these folds. Namely, SARAF_L lacks the Greek-key motif of Xenavidin and Avidin-related protein 2, and is not composed of strictly anti-parallel β -sheets as are the γ -COPI appendage domain and the *B. anthracis* conserved domain. Hence, SARAF_L has a novel β -sandwich architecture that we now call the SARAF-fold.

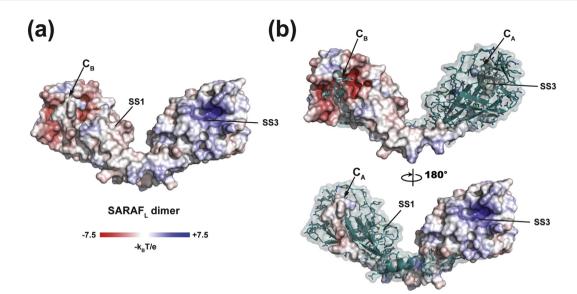


Fig. 2. SARAF_L electrostatic surface potential. (a) Electrostatic surface potential for the SARAF_L dimer calculated in 150-mM ionic strength. (a) Electrostatic surface potential for one monomer of the SARAF_L dimer. In both panels, select elements are labeled.

Analysis of >90 SARAF_L-related sequences uncovers a set of related proteins spanning all five vertebrate classes (mammals, birds, amphibians, fish, and reptiles), including some very ancient animals such as the sea lamprey (*Petromyzon marinus*) and coelacanth (*Latimeria chalumnae*). This family of SARAF_L homologs has strong conservation of the six cysteines that form the three SARAF_L disulfides as well as high conservation of many residues that form the core of the SARAF_L fold (Figs. 1b, 4a, and S2). We did not find SARAF-like sequences among other metazoans, but, surprisingly, identified a group of transmembrane proteins similar to SARAF_L in fungi, including organisms that are pathogens of mammals, insects, or plants (Fig. S3). This group of SARAF_L han

Protein (PDB)	Z-score	RMSD (Å)	Strand order	Structure
SARAF		_		The second second
γ-COPI (1R4X)	2.3	2.9	រ៉ាញារុ	E
Xenavidin (2UYW)	2.2	4.1	រ៉ោហា	6
Avidin related protein 2 (1WBI)	2.1	4.1	រ៉ោហា	
Bacillus anthracis conserved domain (3FBQ)	2.0	3.6	ព្រៃព្រំ	

Fig. 3. Analysis of the SARAF_L fold. SARAF_L structural homologs identified using DALI [28]. DALI search *Z*-score, RMSD_{Ca}, diagram of strand topology, and structure are shown. Cartoon representations highlighting secondary structure elements are colored by rainbow from N-terminus (blue) to C-terminus (red).

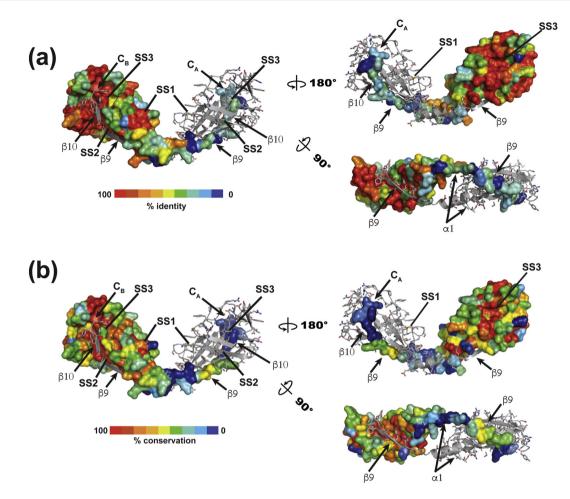


Fig. 4. Conservation mapping of vertebrate sequences on the SARAF_L structure for (a) vertebrate and (b) fungal sequences. One member of the dimer is shown in surface rendering. Select structural elements are labeled.

the vertebrate sequences. For example, the exposed disulfide, SS1, lacks one of the cysteines in a small subset of the fungal sequences, and the swapped $\beta 9/\beta 10$ strand is not well conserved. Nevertheless, the data clearly identify the presence of key elements of the core SARAF_L structure (Fig. 4b). The unique nature of the SARAF_L structure, together with the presence of clear homologs in vertebrates and fungi, indicates that SARAF_L structure represents a previously unknown, widely-occurring protein fold.

$\mathsf{SARAF}_\mathsf{L}$ dimerization depends on the swapped domain

The presence of the domain-swapped dimer in the crystals prompted us to investigate the nature of this interaction further. Characterization by size exclusion chromatography and multi-angle light scattering (SEC-MALS) [29] indicated the dominant presence of SARAF_L monomers in solution at 64 μ M (observed 15.84 ± 0.08 kDa, calculated 15.49 kDa) (Fig. 5a). Further probing with glutaraldehyde cross-linking revealed a dimeric species that appeared

with increasing protein concentrations into the 100-µM range (Fig. 5b), suggesting that the propensity to dimerize is weak. As the domain-swapped dimer observed requires an exchange of β 9 and β 10 (Fig. 1c and d), we created a SARAF₁ deletion construct truncated at residue 150 to remove the domain-swapped β -strands β 9 and β 10 ($\Delta\beta$ 9/ $\Delta\beta$ 10). Expression and purification of SARAF_L($\Delta\beta$ 9/ $\Delta\beta$ 10) yielded a protein having similar properties to SARAF₁ running as a monomer on gel filtration (Fig. S4a and b) and having a similar circular dichroism spectrum to SARAF₁, indicative of a folded protein (Fig. S4c). Notably, the deletion of the domain-swapped strands, β9 and β10, dramatically diminished the ability of the protein to be crosslinked by glutaraldehyde (Fig. 5b), supporting the idea that the dimer seen in SARAF₁ relies on the domain swap interaction of $\beta 9$ and $\beta 10$.

To probe the strength of SARAF_L dimer formation, we used equilibrium analytical ultracentrifugation. Fits of the SARAF_L data using a single-species monomer model yielded upwardly curving residuals, particularly at the highest concentration (200 μ M),

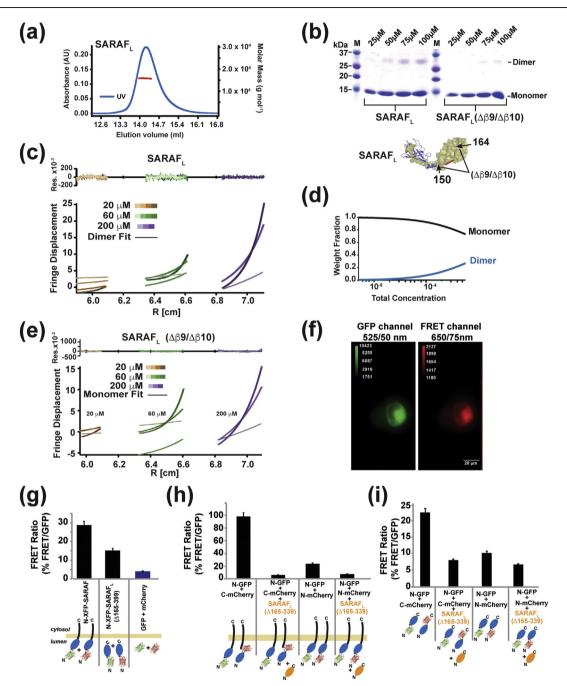


Fig. 5. SARAF self-association requires the SARAF luminal switch domain. (a) SEC-MALS of 64 μM (1 mg ml⁻¹) SARAF_L (experimental Mw 15.84 kDa ± 0.08, Mw/Mn = 1.000 ± 0.007, predicted monomer Mw 15.49 kDa). (b) SDS-PAGE of glutaraldehyde crosslinked SARAF_L and SARAF_L ($\Delta\beta9/\beta10$) as a function of protein concentration indicates SARAF_L dimerization. (c) Equilibrium ultracentrifugation of SARAF_L at the indicated concentrations. Rotor speeds of 10K, 18K, 22K, and 31K rpm are denoted by increasingly darker shades for each concentration. Residuals show fits to a monomer–dimer self-association model. (d) Calculated fraction of monomer and dimer SARAF_L species as a function of concentration. (e) Equilibrium ultracentrifugation of SARAF_L ($\Delta\beta9/\beta10$) at the indicated concentrations. Rotor speeds of 10K, 18K, 22K, and 31K rpm are denoted by increasingly darker shades for each concentration. Residuals show fits to a single species model. (f) Example of images taken of cells expressing GFP-SARAF and mCherry-SARAF using DualView. The red channel is the FRET signal. (g) Bar graph describing the FRET/GFP signal ratio of acquired images as described in panel f. Fluorescent tags are fused N-terminally to SARAF or SP-SARAF_L-R [SARAF luminal domain ($\Delta165$ –339) with a signal peptide, SP, and an ER retention signal, R]. Signals from cells expressing monomeric SP-GFP-R and SP-mCherry-R are used to establish the background signal. (h) SP-SARAF_L-R (orange) inhibits the FRET signals between XFP-SARAF and (i) N-terminally tagged, SP-XFP-SARAF_L-R, or C-terminally tagged, SP-SARAF_L-XFP-R, luminal domains. For panels g–i, *** denotes *p* < 0.001. n for each combination is denoted above the bars.

that were indicative of a poor fit and the formation of a higher-order species (Fig. S4d). Accordingly, the SARAFL data could be well fit with a monomerdimer association model (Fig. 5c and d), as indicated by the uniformly stochastic residuals. This analysis yields an estimate of the SARAFL dissociation constant in the low millimolar range ($K_d \sim 2$ mM). Such a value is entirely in line with the observation that SARAF₁ is monomeric under the low micromolar concentrations conditions used for SEC-MALS (Fig. 5a). In contrast to the behavior of SARAF, equilibrium analytical sedimentation studies of SARAF₁ ($\Delta\beta$ 9/ $\Delta\beta$ 10), which lacks the ability to form domain-swapped dimers, showed that this construct behaved as a monomeric protein that was well fit by a single species model (Fig. 5e). Together with the crosslinking studies, these data demonstrate that the domain swap of the SARAF_L $\beta 9/\beta 10$ element is essential for dimerization.

SARAF self-associates in the ER

Given that purified SARAF₁ forms dimers solution, we sought to probe the extent to which such an interaction might occur in the context of a cell. We transfected HEK293 cells with equal amounts of fulllength SARAF constructs bearing the fluorescent proteins, green fluorescent protein (GFP) [30] or mCherry [31], fused to the SARAF N-terminus (GFP-SARAF and mCherry-SARAF) and measured the FRET between the two constructs. We determined the amount of FRET by measuring the fluorescence emitted from mCherry under exclusive excitation of GFP, using Dual-View imaging, and guantified as the ratio of the red to green fluorescent intensities (FRET signal) (Fig. 5f). We then used a similar approach with N-terminally-tagged soluble luminal domains SARAF(Δ 165-339) that lacked the transmembrane anchor and that were targeted to the ER lumen by bearing both the SARAF signal peptide (SP) and a C-terminal retention signal (KDEL). FRET signals from the GFP-SARAF($\Delta 165-339$) and mCherry-SARAF(Δ165–339) pair were substantially smaller than the GFP-SARAF and mCherry-SARAF pair but were still well above background (Fig. 5g). These results suggest that, in line with the biochemical studies, the SARAF luminal domains selfassociate in the ER. This association happens whether the luminal domain is soluble form confined to the ER or is membrane anchored. The stronger FRET signals from the full-length constructs indicate that membrane anchoring enhances the luminal domain effective concentration [32,33] and facilitates self-association.

Because of the domain-swapped SARAF_L dimer architecture, there is a much shorter distance between the N-and C-termini of the dimer partners (19.6 Å, Cα–Cα) than between the N- and C- termini of an individual subunit (66.7Å, Cα–Cα) or the N- and C-termini of the dimer partners (75.4 Å and 61.8 Å, respectively) (Fig. S4e). These constraints predict that FRET signals will be larger between constructs in which the fluorophores are placed on opposite termini of the tested pairs (i.e., N-donor and Cacceptor). In line with this prediction, FRET between the GFP-SARAFL:SARAFL-mCherry pair was ~3fold larger than the FRET signals observed from coexpressed pairs having each fluorophore fused to the SARAF₁ N-terminus, GFP-SARAF and mCherry-SARAF (Fig. 5h). Furthermore, in both cases, coexpression of untagged SARAF($\Delta 165-339$) reduced the FRET signals to background levels, indicating that the FRET signals derive from co-association of the test proteins (Fig. 5i). Together, these data strongly support the notion that the domain-swapped form of the SARAF₁ occurs in a cellular context, forms in the full-length protein in cell membranes, that similar to in solution, self-association is reversible.

Design and characterization of SARAF_L "Cys-lock" mutants identifies the SARAF luminal switch domain

The crosslinking and sedimentation equilibrium studies suggest that domain-swapped dimer exchanges freely with the monomeric state. In order to characterize properties of the monomer and dimer forms separately, we set out to create a SARAFL mutant that would be incapable of domain swap. We reasoned that incorporation of a fourth disulfide bond between the swapped strand and the core of SARAF₁ structure could serve as a "Cys-lock" that would covalently tether the swapped 69/610 strand to the body of the protein. We identified a residue pair, Lys98–Ala156 on strands ß6 and ß9, as having favorable geometry to form such a disulfide when each member was mutated to cysteine. Purification of SARAF_L K98C/A156C by ion exchange chromatography revealed the presence of two species corresponding to monomer and dimer forms present in a ratio of ~10:1 (Fig. S4f) and having different mobilities on size exclusion chromatography (Fig. 6a). SDS-PAGE under non-reducing conditions revealed two, separate species that ran at molecular weights consistent with the monomeric form (15.5 kDa) and the dimeric form (31.0 kDa). By contrast, both species ran identically upon addition of reducing agent (Fig. 6b), indicating that the two forms are the disulfide linked dimer (16.5 ml form) and disulfide linked monomer (18.3 ml form), respectively (Fig. 6a).

Both SARAF_L K98C/A156C forms produced crystals that diffracted X-rays to high resolution, 1.58 and 2.1 Å for the monomer and dimer, respectively (Table S1). Molecular replacement using the SARAF_L core lacking the surface loops and the $\beta 9/\beta 10$ strand revealed clear electron density in both structures for the engineered K98C/A156C "Cys-lock"

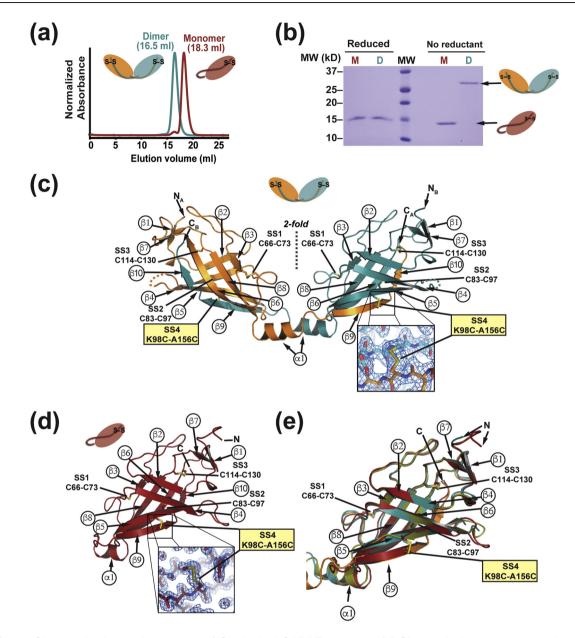


Fig. 6. Characterization and structures of Cys-locked SARAF_L mutants. (a) Size exclusion chromatography profiles monomer and dimer species of SARAF_L K98C/A156C. The Cys-locked dimer species elution profile is shifted earlier, consistent with a larger hydrodynamic radius. (b) SDS-PAGE gel of purified SARAF_L K98C/A156C monomer (M) and dimer (D) species. The dimer band collapses to the same size as the monomer band upon addition of reducing agent (β ME). (c) Crystal structure of the Cys-locked SARAF_L K98C/A156C dimer showing the same topology and disulfide bonding as wild-type SARAF_L. Inset shows electron density (blue mesh, 1.5 σ) for the engineered extra fourth disulfide bond (SS4). (d) Crystal structure of the Cys-locked SARAF_L K98C/A156C monomer again showing the same strand arrangement and topology as wild-type SARAF_L with the exception of β 9 and β 10 inserting in cis into the monomer β -sheet. Inset shows electron density (blue mesh, 1.5 σ) for the engineered (SS4). (e) Superposition of wild-type SARAF_L (smudge green), Cys-locked SARAF_L K98C/A156C dimer protomers (orange and deep teal), and Cys-locked SARAF_L K98C/A156C monomer (firebrick red) showing the fold conservation across all three structures.

disulfide (Fig. 6c and d). Model building and refinement revealed that the Cys-lock monomer and Cys-lock dimer protomer maintain the same overall fold as the wild-type SARAF_L dimer protomer (Fig. 6e) (RMSD_{Ca} = 0.739 and 0.590 for residues 33–145 of the Cys-lock monomer and Cys-lock dimer *versus*

SARAF_L, respectively). Notably, the SARAF_L K98C/A156C Cys-lock monomer has the marked difference that the tail, which extends from the core in the dimeric form, is wrapped under the bottom of SARAF_L so that $\beta \beta/\beta 10$ inserts in *cis* to complete the anti-parallel and mixed β -sheets of the SARAF_L fold

(Fig. 6d). The α_1 helix is retained despite this topology change of the SARAF_L tail, although it is shorter by one helical turn relative to the dimer form. In both the Cys-locked dimer and the Cys-locked monomer, the electrostatic surface potentials were very similar to wild-type SARAF_L (Fig. S5). These structural studies demonstrate that the switch from monomer to dimer requires changes only in the $\beta 9/\beta 10$ tail and not the SARAF_L core. Accordingly, we term the $\beta 9/\beta 10$ tail as the "SARAF luminal switch domain."

SARAF dimerization affects SOCE inactivation

To test the functional importance of the SARAF luminal switch, we used whole-cell patch clamp to measure SOCE currents from HEK293 cells that were co-transfected with Orai1-CFP and STIM1mCherry along with GFP tagged version of SARAF, SARAF-GFP, or a SARAF mutant lacking the luminal switch, SARAF ($\Delta\beta9/\beta10$)-GFP. Recording conditions included 1,4-dihydroxy-2,5-di-*tert*butylbenzene, a reversible SERCA inhibitor, to allow for both depletion and refilling of Ca²⁺ stores. Because we observed a direct relationship between STIM1 expression level and Orai1 current density (Fig. S6), we limited our analysis to cells expressing STIM1-mCherry at or below a threshold of 5000 fluorescence counts/cell to ensure that any functional effects were not due to exceptionally high levels of STIM1. Cells expressing SARAF-GFP or SARAF $(\Delta\beta9/\beta10)$ -GFP had comparable levels of STIM1 expression (Fig. 7a and b) and similar passive membrane properties. However, we observed that the SOCE current densities 18s after addition of external Ca²⁺ to activate the current were roughly twice as large in cells expressing SARAF($\Delta\beta9/\beta10$)-GFP versus SARAF-GFP [42.6 \pm 7.5 pA/pF (n = 9) and $21.2 \pm 4.3 \text{ pA/pF}$ (n = 11), p < 0.05, respectively] (Fig. 7c). Furthermore, the time to maximum response for SOCE current activation was faster by ~2-fold in cells expressing the SARAF luminal switch mutant $[17.1 \pm 1.7 \text{ s and } 32.5 \pm 4.6 \text{ s } (n = 11)$ and (n = 9), p < 0.01, for SARAF ($\Delta\beta 9/\beta 10$)-GFP and SARAF-GFP, respectively] (Fig. 7d). These results indicate that the self-associated form of SARAF accelerates SOCE inactivation and that domain swap of the luminal switch is important for stabilizing this state.

Discussion

SARAF is a transmembrane, ER-resident, negative regulator of SOCE [24]. Understanding how it influences SOCE has been limited due to its lack of similarity to proteins of known structure. Crystallographic determination of the structure of the SARAF luminal domain, SARAF_L, shows that this domain comprises a novel, 10-strand β -sheet fold constrained by three conserved disulfides that we name the "SARAF-fold." Although, the SARAF fold belongs to a class of β -sheet sandwich proteins represented by the y-COPI appendage domain (1R4X), Xenavidin (2UYW), Avidin-related protein 2 (1WBI), and a *B. anthracis* conserved domain (3FBQ) (Fig. 3), the SARAF fold and topology are unique. Homologs having all six cysteines that form the three SARAF_L disulfides as well as many conserved core residues in the SARAFL core occur in all five vertebrate classes (mammals, birds, amphibians, fish, and reptiles), including guite ancient members of this phylum, such as the sea lamprey (P. marinus) and coelacanth (L. chalumnae) (Figs. 1b and 4a and S2). Although SARAF, is widespread among vertebrates, it appears to be absent from other metazoans. Intriguingly, we found a set of fungal transmembrane proteins that are also SARAF_L homologs (Figs. 4b and S3). This unusual distribution of homologs in both vertebrates and fungi establishes that the previously unknown SARAF-fold is widespread. Because fungi are not known to use the SOCE pathway, it seems likely that the SARAF-fold has other functions beyond its role in SOCE regulation.

Apart from its unique fold, our structural studies revealed a second key feature of the SARAF_L fold, the ability to domain-swap. The formation of intertwined protein assemblies by exchange of identical structural elements is observed in many classes of soluble and transmembrane proteins [34–37]. Although this phenomenon provides a straightforward mechanism for homo-oligomer formation, its functional relevance is often not clear [34,37,38]. In SARAF_L, the swapped domain is a simple element comprising the β 9 and β 10 β -strands that insert into the essentially rigid, stable core of the rest of the fold.

Two structure-based protein design strategies firmly establish that the $\beta 9/\beta 10$ strands form the dimerization element. First, design of SARAF_L mutant lacking β9 and $\beta 10$ yielded a well-folded, stable protein that only differed from SARAF_L in its inability to dimerize. Second, structure-based design to incorporate a disulfide between $\beta 9$ and the SARAF_L core yielded two covalently trapped species, a covalent dimer and a self-ligated monomer. Structural studies show that apart from the topological change, these two forms are identical. The intrinsic affinity of SARAF luminal switch-mediated dimerization is modest having a dissociation constant in the low millimolar range $(K_{\rm d} \sim 2 \text{ mM})$. Nevertheless, the transmembrane nature of full-length SARAF clearly imposes diffusional restrictions that serve to increase the effective concentration [32,33] and favor self-association (Fig. 5g). Importantly, overexpression of nonmembrane anchored SARAF_L is able to suppress the amount of dimer, indicating that full-length SARAF self-association is reversible (Fig. 5h). Hence, we term the β9/β10 element as the "SARAF luminal switch" domain, as biochemically this is the sole element

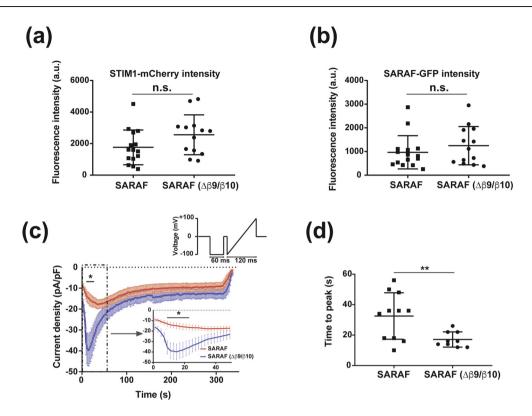


Fig. 7. Self-associated SARAF accelerates SOCE inactivation (a) STIM1-mCherry and (b) SARAF-GFP expression measured by fluorescence using epifluorescence illumination of cells expressing Orai1-CFP, STIM1-mCherry and SARAF-GFP, or SARAF (Δβ9/Δβ10)-GFP. (c) Average La³⁺-sensitive current densities of HEK293 cells expressing Orai1-CFP, STIM1-mCherry, and SARAF-GFP or SARAF (Δβ9/Δβ10)-GFP measured at the end of 60-ms hyperpolarizing pulse to – 100 mV in the presence of 10 mM extracellular Ca²⁺. Inset shows the first 50 s of recording. Stimulation protocol is shown and was applied at 0.5 Hz. (d) Time to peak measured following the application of 10 mM Ca²⁺ for SARAF and SARAF (Δβ9/Δβ10). n = 11 and 9, respectively. **p* < 0.05; ***p* < 0.01; n.s., not statistically different. Error bars indicate s.e.m.

required to convert between monomeric and dimeric forms and this domain is necessary for self-association of SARAF in membranes.

A hallmark of the SOCE pathway is that it is governed by reversible self-association of the ER sensor, STIM, in response to changes in ER calcium levels [15,17-22]. SARAF functional studies indicate that domain-swap mediated self-association is key for the ability of SARAF to accelerate inactivation of the SOCE current (Fig. 7). Whether, as with STIM1, SARAF also forms higher-order oligomers remains to be elucidated. In this regard, it is worth noting that crystal packing of SARAF, shows a simple faceto-face arrangement in which the C-termini that link SARAF, to the transmembrane portion all face the same direction (Fig. S1b). Such an arrangement would be compatible with formation of higher-order assemblies of SARAF dimers in the context of the membrane. Nevertheless, although the self-associated form of SARAF, is a dimer in both the crystal structure and in solution, non-symmetric domain swapping via the SARAF luminal switch could also form higher-order domain-swapped assemblies as has been observed in other domain-swapped proteins [38]. Hence, although SARAF clearly self-associates in the membrane

(Fig. 5g–i), and this association is important for function, the stoichiometry of self-organization and how such assemblies might be affected by interactions with STIM or Orai requires further investigation.

Initial characterization of SARAF demonstrated that it acts in a calcium-dependent manner, inhibiting SOCE only when ER stores were refilled with calcium [24]. SARAF_L has no identifiable Ca²⁺-binding motifs, and despite being crystallized in 1 mM CaCl₂, there is no evidence for non-canonical binding sites for Ca²⁺ on SARAF_L. Hence, how SARAF senses and responds to ER Ca²⁺ changes remains to be discovered. Given the absence of Ca²⁺ binding to SARAF_L, it may be that there is some type of Ca²⁺-mediated interaction of SARAF_L with the membrane inner leaflet or Ca²⁺-dependent control of SARAF by a yet to be defined ER Ca²⁺sensor protein.

SOCE is a complex, multicomponent process that involves the reversible, coordinated association of proteins in both the ER and PMs [15]. The ER resident transmembrane protein SARAF serves an important role in this phenomenon by fine-tuning SOCE activity in response to ER refilling with Ca²⁺. The discovery that the SARAF luminal domain has a unique, disulfide-bonded β -sheet protein fold, capable of domain-swap mediated self-assembly that impacts function sets a key structural framework for understanding the basic roles of SARAF in controlling SOCE and the possible roles for SARAF in cancer, neurodegenerative diseases, and cardiomyopathy [39–43].

Materials and Methods

Protein expression and purification

Human SARAF_L (residues 30-164) or SARAF_L $(\Delta\beta9/\Delta\beta10)$ (residues 30–150) were cloned into a modified version of the pET28 vector containing an N-terminal combination His₆ and maltose binding protein tag followed by TEV protease site (HMT). Point mutants were introduced using site-directed mutagenesis. The SARAF₁ or SARAF₁ ($\Delta\beta9/\Delta\beta10$) constructs were transformed into SHuffle Express cells (NEB) and grown in 1 L cultures of 2YT media at 37 °C. The SHuffle Express cells have deletions of the genes for glutaredoxin reductase and thioredoxin reductase ($\Delta gor \Delta trxB$), which allow disulfide bonds to form in the cytoplasm. In addition, they constitutively express a chromosomal copy of the disulfide bond isomerase DsbC allowing for rearrangement of improperly oxidized disulfides. Cultures were induced with 0.5 mM IPTG at an OD_{600nm} of ~0.6 and moved to 24 °C. Protein was expressed overnight, and cells were harvested by centrifugation and then immediately flash frozen in liquid nitrogen and stored at - 80 °C. Frozen cell pellets were thawed on ice and resuspended in lysis buffer [25 mM HEPES (pH 7.4), 300 mM KCl, 1 mM CaCl₂, 10 mM Imidazole, 1 mM PMSF, 1 µg ml⁻¹ DNasel] at a ratio 6 ml lysis buffer to 1 g cell pellet. Resuspended cells were disrupted by sonication and insoluble material pelleted by centrifugation. Clarified lysate was mixed with 2 ml bed volume of Talon beads (Clontech) and incubated while rocking for 1 h at 4 °C. Following incubation, beads and lysate were transferred to a gravity flow column at washed with 2 × 30 ml lysis buffer before eluting with 15 ml lysis buffer containing 400 mM imidazole. After elution, protein was digested overnight with TEV at 4 °C and buffer exchanged into 10 mM Tris (pH 8.8) 10 mM KCl over a HiPrep desalting column. Desalted SARAF was then passed over a POROS MC20 column followed by an Amylose column to remove TEV and cleaved MBP. SARAF was further purified by MonoQ ion exchange before final gel filtration in 10 mM HEPES (pH 7.4), 200 mM KCl, and 1 mM CaCl₂ and concentrated to 10 mg ml^{-1} for crystallization.

The double mutant SARAF_L K98C/A156C was expressed and purified following the same protocol as wild-type protein with the separation of monomer and dimer populations occurring at the ion exchange

step before proceeding on to gel filtration. SARAF 31-164 K98C/A156C monomer and dimer were concentrated to 10 and 4 mg ml⁻¹, respectively, prior to crystallization.

Crystallization and data collection

Crystals used for structure determination of the wildtype construct were grown in 0.1 M BisTris (pH 6.5) and 18%-20% PEG 5000 MME in hanging drop format. Crystallization drops were set over a thin layer of vacuum grease to prevent crystals from sticking to the coverslips and facilitate harvesting. Harvested crystals were cryoprotected by sequential soaks in mother liquor plus 5%, 10%, 15%, and 20% glycerol before flash cooling in liquid nitrogen. For experimental phasing, crystals were soaked with 1 mM K₂Pt (NO₂)₄ overnight before back-soaking into mother liquor and cryoprotecting in glycerol as above. Crystals of SARAF 30-164 K98C/A156C monomer were grown in 0.1 M sodium acetate (pH 4.2-4.6) and 1.2-1.6 M sodium formate in standard hanging drop format without the use of vacuum grease. Harvested crystals were cryoprotected by sequential soaks in mother liquor plus 5%, 10%, 15%, and 20% glycerol before flash cooling in liquid nitrogen. Crystals of SARAF 30-164 K98C/A156C dimer were grown in 3%–5% glycerol or ethylene glycol after first treating protein to mild heating at 37 °C for 5 min or ultracentrifigation (40,000 rpm, 30 min, 4 °C) to remove microcrystals that spontaneously form in concentrated protein solution. Harvested crystals were cryoprotected by directly soaking in 25% ethylene alvcol before flash cooling in liquid nitrogen.

Data were collected at Advanced Light Source beamline 8.3.1. Native data sets of wild-type SARAF were collected at a wavelength of 1.127 Å and diffracted to 1.90 Å. Peak, inflection, and high remote data sets were collected from $K_2Pt(NO_2)_4$ -soaked crystals and diffracted to a resolution of 2.15 Å. Native data sets for both SARAF K98C/A156C monomer and dimer were collected at a wavelength of 1.116 Å and diffracted to resolutions of 1.58 and 2.10 Å, respectively.

Data processing and structure determination

Data from both native and $K_2Pt(NO_2)_4$ -soaked wild-type crystals were indexed, integrated, and scaled in spacegroup C2 using autoPROC [44]. autoSHARP [45] was used to determine initial experimental phases using peak, inflection, and remote data sets from $K_2Pt(NO_2)_4$ -soaked crystals finding a total of 10 platinum sites. An initial model was obtained using ARP/wARP [46] and improved with iterative rounds of manual rebuilding with COOT [47] and refinement with Phenix [48]. The SARAF K98C/A156C monomer and dimer structures were phased by molecular replacement with PHASER

[49] using the wild-type structure with the tail and surface loops removed as a search model. As with the wild-type structure, the models were improved with iterative rounds of manual rebuilding in COOT [47] and refinement in Phenix [48].

Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed at 4 °C in an Optima XL-I analytical ultracentrifuge (Beckman Coulter). Prior to loading the rotor cells, 500 μ l of SARAF_L or SARAF_L($\Delta\beta9/\Delta\beta10$) was dialyzed against 1 L of buffer [200 mM KCl, 10 mM HEPES, (pH 7.4)] overnight at 4 °C. 125 µl of SARAF or SARAF_I ($\Delta\beta9/\Delta\beta10$) were loaded into six chamber center pieces at three concentrations of 20, 60, and 200 µM determined by absorbance at 280 nm [50]. Dialysate buffer (115 µl) was loaded into adjacent reference chambers. Data were acquired using interference optics at rotor speeds of 10K, 18K, 22K, and 31K rpm. Data acquired at multiple loading concentrations and rotor speeds were modeled globally in IgorPro using a standard monomer-dimer self-association model. V_{bar} and solvent density were calculated using Sednterp, and the interference extinction coefficient was calculated using the formula $e_{int} = 2.733 \times MW$. For global fitting, Vbar, MW, solvent density, and e_{int} were held constant, while K_d (for N = 2) was allowed to float.

Glutaraldehyde crosslinking

Purified SARAF_L or SARAF_L($\Delta\beta9/\Delta\beta10$) in a buffer of 10 mM HEPES, (pH 7.4) and 200 mM KCI at concentrations ranging from 25 to 100 µM, determined by absorbance at 280 nm [50], was combined with 0.01% glutaraldehyde (Aldrich) in a final volume of 10 µl and incubated at room temperature for 10 min. Reactions were quenched by adding 1 µl of 1 M Tris (pH 8.0) to achieve a final concentration of 100 mM and Tris (pH 8) and were then boiled for 10 min in reducing SDS-sample buffer prior. Samples were subsequently analyzed by SDS-PAGE.

SEC-MALS

Multi-angle light scattering (MALS) experiments were carried out at 4 °C using an HPLC (Shimadzu) with UV detector connected to a miniDAWN TREOS MALS detector and an Optilab T-rEX refractometer (Wyatt Technology). One hundred microliters of 1 mg ml⁻¹ of purified SARAF_L was injected onto a Superdex S200 10/300 GL column (GE Healthcare) equilibrated in 200 mM KCl and 10 mM HEPES, (pH 7.4), and eluted peak was detected online. Molecular weight was calculated at each time point during elution using a combination of UV absorbance, light scattering, and differential refractive index measurements with the Astra software package (Astra 6.0, Wyatt Technology). The experimentally determined molecular weight of SARAF_L of 15.84 kDa ($\pm 0.509\%$) compares well with the 15.49 kDa calculated from the protein sequence. SARAF_L was monodisperse with a polydispersity ratio (Mw/Mn) of 1.000 ($\pm 0.719\%$).

Circular dichroism

Prior to measurement, 600 µl each of 10 µM SARAF₁ and 10 μ M SARAF₁ ($\Delta\beta$ 9/ β 10), determined by absorbance at 280 nm [50], were dialyzed overnight at 4 °C against 1 L of 30 mM sodium phosphate buffer. Circular dichroism spectra were measured with a 1-mm path-length quartz cuvette using an Aviv model 215 spectropolarimeter (Aviv Biomedical) equipped with a Peltier temperature controller. Wavelength scans from 320 to 185 nm were taken at 1-nm intervals at 4 °C. Each scan was performed in triplicate from the same sample and subtracted by the average of a triplicate scan of the dialysate for a matched buffer blank. Molar ellipticity was calculated as follows: $\theta = 100(\Delta m)/(Cnl)$, where Δm is the CD signal in millidegrees after buffer subtraction, C is the millimolar peptide concentration, *n* is the number of residues in the peptide, and *l* is the cuvette path length in centimeters.

Cell culture

HEK293 cells were grown in DMEM supplied with fresh L-glutamine, sodium pyruvate, FBS, and penicillin/streptomycin. Cells were split to 35-mm plates 1 day prior to transfection. Transfection with 600 ng of C-terminally GFP-tagged SARAF or SARAF($\Delta\beta9/\beta10$), 200 ng of C-terminally mCherry-tagged STIM1, and 200 ng of C-terminally CFP-tagged Orai1 with PEImax reagent (Polysciences) was performed 22–36 h prior to imaging and electrophysiological experiments. Cells were transferred to poly-L-lysine covered 24-mm coverslips one night prior to the experiments.

FRET

Cells were excited with 470/40-nm light for FRET measurements, and fluorescent signals were collected through the objective and split using Dual-View device (565LP dichroic) to GFP (525/50) and mCherry (650/75) channels using EMCCD 512x512 (Princeton Instruments). Images were processed using SlideBook (Intelligent Imaging Innovations) software and exported as Microsoft Excel files. FRET signals were assessed by dividing the FRET (mCherry) channel by the GFP channel. GFP or mCherry was fused to the N-termini of SARAF_L (XFP-SARAF_L) or SARAF full length (XFP-SARAF) using the following sequence: SARAF(M1–G30)-TG-XFP-RT-SARAF(W31–K164)-KDEL or SARAF(M1–G30)-TG-XFP-RT-SARAF(W31–R339), respectively.

Fusions of monomeric forms of GFP [30] or mCherry [31] were made at the end of the luminal domain (K164) of the SARAF_L (SARAF_L-XFP) or full-length SARAF and were at position K164 using the following s e q u e n c e : S A R A F (1 – 1 6 4) - T G RPACKIPNDLKQKVMNH-XFP-KDEL or SARAF (M1–K164)-TGLGGGGSGGGGGGGGGGGGGAAAR-P A C K I P N D L K Q K V M N H - X F P -LGGGGSGGGGGGGGGGGGAAASGLRS-SARAF (M173–R339), respectively. ER retention signal (KDEL) was added at the C-termini of all luminal SARAF constructs.

Electrophysiological recordings

Membrane currents were recorded under voltageclamp conditions using the whole-cell patch-clamp configuration using an Axopatch 200B (Axon Instruments) amplifier. Patch pipettes were fabricated from borosilicate glass capillaries (2–5 M Ω). Signals were analog filtered using a 2-kHz low-pass Bessel filter. Data acquisition and analysis were done using pCLAMP 9 software (Molecular Devices). Current densities were calculated by normalizing currents to cell capacitance. The recording protocol consisted of a 60-ms hyperpolarizing step to -100 mV from 0 mV, followed by a 20-ms step to 0 mV, and a 120-ms ramp from - 100 to + 100 mV repeated at 0.5 Hz for 300 s. All data were leak-corrected using the current in lanthanum-containing solution. The EGTA (ethylene glycol-bis(β -aminoethyl ether)-N, N, N', N'-tetraacetic acid) internal solution contained 150 mM Cs aspartate, 8 mM MgCl₂, 1.2 mM EGTA, 10 mM HEPES, and 2 mM Mg-ATP. The pH was titrated to pH 7.2 with CsOH. The external solution contained 145 mM NaCl, 2 mM MgCl₂, 2.8 mM KCl, 10 mM CsCl, 10 mM HEPES, and 10 mM glucose. The pH of the external solution was titrated to pH 7.4 with NaOH. CaCl₂ (10 mM) and 100 µM EDTA (ethylenediaminetetraacetic acid) or 10 mM MgCl₂ and 1 mM EDTA were added to the external solution for high-Ca²⁺ or Ca-free solution, respectively.

Fluorescence was measured from images acquired using EMCCD 1024 \times 1024 iXonUltra camera under epifluorescence, and analyzed with VisiView software (Visitron Systems GmbH). Cells were excited using Xcite Exacte (Excelitas technologies) using the following filters: excitation filters ET470/40 for GFP and FF01-580/14 for mCherry; Emitted light was filtered with BP536/40 for GFP and HQ650/75 for mCherry. Data are presented as mean \pm S.E.M.

Accession numbers

Coordinates and structure factors for SARAF_L, SARAF_{L.SS} monomer, and SARAF_{L.SS} dimer are deposited with the RCSB under accession codes 602U, 602V, and 602W, respectively, and will be released immediately upon publication.

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Author Contributions: C.K., A.M., E.R., and D.L.M. conceived the study and designed the experiments. C.K. cloned, expressed, purified, crystalized, and determined the structure of SARAF_L and variants and performed biochemical and biophysical characterization experiments. A.M., R.P., and A.R. performed electrophysiology and imaging experiments. A.M., R.P., A.R., I. K., and E.R. analyzed the imaging and electrophysiology data. A.M. performed molecular biology experiments. E.R. and D.L.M analyzed data and provided guidance and support. C.K., A.M., R.P., E.R., and D.L.M. wrote the paper.

Declaration of Competing Interest: The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2019.05.008.

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†Equal contributions.

Abbreviations used:

SOCE, Store-Operated Calcium Entry; CRAC, calcium release-activated channel; PM, plasma membrane; ER, endoplasmic reticulum; SARAF, SOCE-associated regulatory factor; FRET, Förster resonance energy transfer; SEC-MALS, size exclusion chromatography and multi-angle light scattering; GFP, green fluorescent protein.

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Supplementary Material for:

SARAF luminal domain structure reveals a novel domain-swapped β -sandwich fold important for SOCE modulation

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^{26 Feb 19} **Fig. S1**

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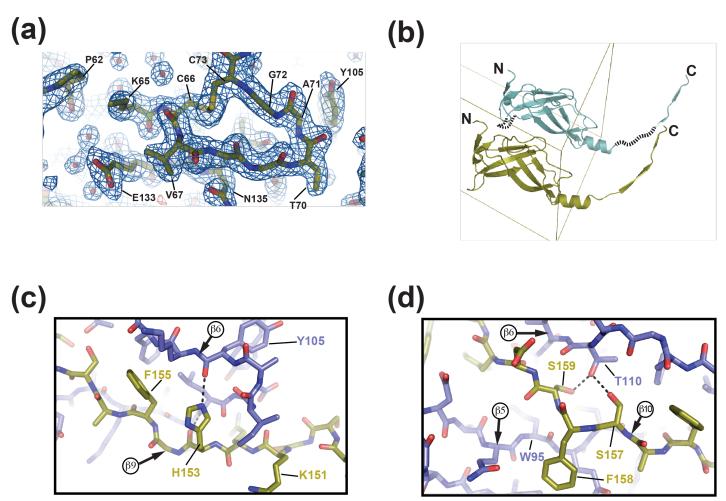


Fig. S1 X-ray crystal structure of SARAF_L (a) Exemplar $2F_{o}$ - F_{c} electron density for SARAF_L (blue mesh) contoured at 1σ . Select residues are indicated. Model shows the final refined structure. (b) SARAF_L unit cell containing two SARAF_L molecules (deep olive and teal). Missing loops of the teal copy are indicated by black dashed lines. (c) and (d) Key interactions of domain swapped strands (c) $\beta9$ (deep olive) and (d) $\beta10$ (deep olive) with the SARAF_L core (slate).

26 Feb 19 _{Fig. S2}

	β1	β2 β:	3 β4	β5	β6	β7	β8 α1	β9	β10			тм
Class UNIPROT Organism			•	552								******
5		r				ss3	_					
M Q96BY9 Homo sapiens M G3H619 Cricetulus griseus	GWNDP-DRMLLRD	VKALTLHYDRYTTSRRLDPIPQLK	VGGTAGCDSYTPKVIQCQ	NKGWDGYDVQWECKTDLDI	AYKFGKTVVS	EGYESSEDQYVL	RGSCGLEYNLDYTELGLQKLKE	ESGKQHGFASH	SDYYYK		WSSADSCNMSC	LITIVVLLGIAFVVYKLFLSDGQYS LVTIGVLLAVAYVVYKLFLSDGQAS
M Q6AYN2 Rattus norvegicus	CWKNP-DRILLRD	VEALTLYSDRYTTSRRLDPIPQLKC	VGGTAGCDAYTPKVVQCQI	NKGWDGYDVQWECKTDLDI	AYKFGKTVVS	EGYDSSEDQYIL	RG <mark>SCGL</mark> EYNLDYTELGLSKLKE	ESGKHQSF	?SD <u>YY</u> HK		LSSVDSCGL	-VTVAVLFVLAFVVYKLFLSDGQGS
M Q8R3Q0 Mus musculus	GWNDP-DRILLRD	VKALTLYSDRYTTSRRLDPIPOLKC	VGGTAGCEAYTPRVIOCO	NKGWDGYDVOWECKTDLDI	AYKFGKTVVS	EGYESSEDOYVL	RGSCGLEYNLDYTELGLKKLKE	ESGKHOGF	?SDYYHK		LYSSDSCGF	-ITIAVLFVLAFAVYKLFLSDGOGS
M A0A091ER24 Fukomys damarensis	NKILLRD	VKALTLHHDRYTTSRRLDPIPQLRC	VGGTAG <mark>C</mark> DSYTPQVIR <mark>C</mark> QI	NKGWDGYDVQWE <mark>C</mark> KTDLDI	AYRFGKTVVS	EGYDSSEDQYVL	RGSCGLEYHLDYTEIGLKKLKE	ESGKQQGF-SH	SDYYYK		LYSADSCATGO	LVTIGVLLTIAFVVYKLFLSDGQYS
M AOAOP6K1K6 Heterocephalus glab M G3T5R5 Loxodonta africana	CWNDP-DRILLRD	VKALTLHYDRYTTSRRLDPIPQLRC	VGGTAGCDSYTPKVIQCQ	KGWDGYDVQWECKTDLDI	AYRFGKTVVSC	EGYDSSEDQYVL	RGSCGLEYHLDYTEIGLKKLKE	ESGKQQGF-SH FSGRKHGFNSH	SDFYYK		IYSADSCATGO	LVTIGILLTIAFVVYKLFLSDSQYS
M G1TWB6 Oryctolagus cuniculus	CWNDP-DRVLLRD	VKALTLHYDRYTTSRRLEPIPOLKC	VGGTAGCDAYTPKVIOCO	KGWDGYDVOWECKTDLDV	AYKFGKTVVS	EGYESSEDOYVL	RGSCGLEYNLDYTELGLKKLRE	ESGRHOGF-SH	sdyyyx		LYSADSCGLGG	LVTIVVLLTIAFVVYKLFLSDGOYP
M I3M2Y7 Ictidomys tridecemlinea	GWNDP-DRMLLRD	VKALTLHYDRYTTSRRLDPIPOLKC	VGGTAGCDSYTPKVIOCO	KGWDGY DVOWECKTDLDV	AYREGKTVVSC	EGYESSEDOYVL	RGSCGLEYHLDYTEIGLOKLKE	ESGKHPGF-SF	7SDYYYK		LYSADSCGVG0	LVTIAVLLTIAFVIYKLFLSDGOYS
M HOWN35 Otolemur garnettii M U3E7B2 Callithrix jacchus	GRNDP-DRILLRD	VKALTLHYDRYTTSRRLDPIPQLKC	VGGTAG <mark>C</mark> DAYTPKVIQ <mark>C</mark> QI	NKGWDGYDVQWE <mark>C</mark> KTDLDV	AYKFGKTVVS	EGYESSEDQYVL	RGSCGLEYNLDYTELGLKKFKE	ESGKHHSFAAH	SDYYNK		WYSADACSTGC	LITIVVLLTIAFAVYKLFLSDGQCS
M AOAO96MYKO Papio anubis		VKALTLHYDRYTTSRRLDPIPOLKO										
M A0A0D9RTI6 Chlorocebus sabaeus	CHINDD DD TTTDD	WWAT MT WWDDWMMCDDT DDTDOT WC	UCOMP CONTRACTOR	WOUDOWDUOWDOWD DT	D WWWWWWWWWW	DOVECODDOVIU	DOGOOT DUNT DUMPT OF OWT WE		3 CD VVVVV		MICE DOOM	TETTING LOT S DIRIVER DI CDCOVC
M F7BVH6 Macaca mulatta	GWNDP-DR <mark>MLL</mark> RD	VKALTLHYDRYTTSRRLDPIPQLKC	V <mark>GGTAGC</mark> DSYTPKVIQ <mark>C</mark> QI	NKGWDGYDVQWE <mark>C</mark> KTDLDI	AYKFGKTVVS <mark>C</mark>	EGYESSEDQYVL	RG <mark>SCGL</mark> EYNLDYTELGLQKLKE	ESGKQHGFASH	SD <mark>YY</mark> YK		WYSADSCNMGG	LITIVVLLGIAFVVIKLFLSDGQIS LITIVVLLGIAFVVIKLFLSDGQIS LITIVVLLGIAFVVIKLFLSDGQIS
M I7GEM7 Macaca fascicularis M G3SGX8 Gorilla gorilla gorilla	GWNDP-DRMLLRD	VKALTLHYDRYTTSRRLDPIPQLKC	VGGTAG <mark>C</mark> DSYTPKVIQ <mark>C</mark> QI	NKGWDGYDVQWECKTDLDI	AYKFGKTVVS	EGYESSEDQYVL	RGSCGLEYNLDYTELGLQKLKE	ESGKQHGFASH	SDYYYK		WYSADSCNMGG	LITIVVLLGIAFVVYKLFLSDGQYS LITIVVLLGIAFVVYKLFLSDGQYS
M G3SGX8 Gorilla gorilla gorilla M G7PD50 Macaca fascicularis	GWNDP-DRMLLRD	VKALTLHYDRYTTSRRLDPIPOLKC	VGGTAGCDSYTPKVIQCQ	KGWDGYDVQWECKTDLDI	AYKFGKTVVSC	EGYESSEDOYVL	RGSCGLEYNLDYTELGLOKLKE	SSGKQHGFASE	SDYYYK		WSSADSCNMSG	LITIVVLLGIAFVVYKLFLSDGQYS
M G1RMH3 Nomascus leucogenys	DRMLLRD	VKALTLHYDRYTTSRRLDPIPOLKC	VGGTAGCDSYTPKVIOCO	NKGWDGYDVOWECKTDLDI	AYKFGKTVVS	EGYESSEDOYVL	RGSCGLEYNLDYTELGLOKLKE	ESGKOHGFASH	SD¥¥¥K		WYSADSCNMSG	LITIVVLLGIAFVVYKLFLSDGOYS
M H2PPZO Pongo abelii	GWNDP-DR <mark>MLL</mark> RD	VKALTLHYDRYTTSRRLDPIPQLKC	VGGTAG <mark>C</mark> DSYTPKVIQ <mark>C</mark> QI	NKGWDGYDVQWE <mark>C</mark> KTDLDI	AYKFGKTVVS <mark>O</mark>	EGYESSEDQYVL	RG <mark>SCGL</mark> EYNLDYTELGLQKLKE	ESGKQHGFASH	SD <mark>YY</mark> YK		WYSADSCNMSG	LITIVVLLGIAFVVYKLFLSDGQYS
M H2QVZ5 Pan troglodytes	GWNDP-DRMLLRD	VKALTLHYDRYTTSRRLDPIPQLK	VGGTAG <mark>C</mark> DSYTPKVIQ <mark>C</mark> QI	NKGWDGYDVQWE <mark>C</mark> KTDLDI	AYKFGKTVVSC	EGYESSEDQYVL	RGSCGLEYNLDYTELGLQKLKE	ESGKQHGFASH	SDYYYK		WSSADSCNMSC	LITIVVLLGIAFVVYKLFLSDGQYS LITIVVLLAIAFGVYKLFLGGGODS
M G1PLN4 Myotis lucifugus M L5LTT8 Myotis davidii	GWNDP-NRILLRD	I OALTLHYDRYTTSRRLNPI POLKC	IGGTAGCDTYTPKVIQCQ	KGWDGYDVQWECKTDLDV	AYKFGKTVVSC	EGYESSEDOYVL	RGSCGLEYDLDYTELGLKKLRE	ESGKNNGFNPE	SDYYK		SNSSGISG	LITIVVLLAIAFGVYKLFLGGGQDS
M S7MQ66 Myotis brandtii	GWNDP-NRILLRD	IOALTLHYDRYTTSRRLNPIPOLRC	VGGTAGCDTYTPKVIOCO	NKGWDGYDVOWECKTDLDV	AYKFGKTVVS	EGYESSEDOYVL	RG <mark>SCGL</mark> EYDLDYTELGLKKLRE	ESGKNNGFNPE	?SD¥¥K		SNSSGISC	LITIVVLLAIAFGVYKLFLGGGODS
M F1RX61 Sus scrofa	GWNDP-DR <mark>ILL</mark> RD	IKALTLHYDRYTTSRRLDPIPQLKC	VGGTAG <mark>C</mark> DSYTPKVIQ <mark>C</mark> QI	NKGWDGYDVQWE <mark>C</mark> KTDLDV	AYKFGKTVVS <mark>O</mark>	EGYDSSEDQYVL	RGS <mark>CGL</mark> EYNLDYTELGLRKLRE	ESGENHGFNSH	SN¥¥NK		LYSPDSCSISC	VITIVLLLAIAFGVYKFFLSDGQDP
M W5PKS2 Ovis aries	CWDER-DRILLRD	IKALTLYYDRYTTSRRLDPIPQLKC	VGGTAG <mark>C</mark> DSYTPKVIQ <mark>C</mark> QI	NRGWDGYDVQWE <mark>C</mark> KTDLDV	AYKFGKTVVSC	EGYESSEDQYVL	RG <mark>SCGL</mark> EYQLDYTELGLKKLRF	ESGKEHGFSSH	SNYYNK		LSSPESCSLSC	VVTIVVLLALAFGVYKFFLSDGHES
M Q08E24 Bos taurus M L8HT65 Bos mutus	DRTLLRD	TKALTLYYDRYTTSRRLEPTPOLKC	VGGTAGCDSYTPKVIOCOL	RGWDGY DVOWECKTDLDV	AYKEGKTVVSC	EGYESSEDOYVL	RGSCGLEYOLDYTELGIKKLRF	ESGKEHGENSE	SNYYSK		LYSPESSSLGG	VVTIVVLLAIAFGVYKFFLSDGHES VVTIVVLLAIAFGVYKFFLSDGHES
M L5KDC2 Pteropus alecto	GWNDA-DRILLRD	IKALTLHYDRYTTSRRLDPVPQLKC	VGGTAGCDSYTPKVIQCO	NKGWDGYDVQWECKTDLDV	AYKFGKTVVS	EGYESSEDOYVL	RGSCGLEYDLDYTDLGLKKLRE	ESGKNHGFNSH	SNYYNK		FYSSNSGGISC	LITIVVLLAIAFGVYKLFLSDGQDS
M K9IZZO Desmodus rotundus	GWKDP-DR <mark>ILLRD</mark>	VKALTLHHDRYTTSRRLDPIPQLKC	VGGTAGCDSYTPKVVQCQI	NKGWDGYDVQWECKTDLDI	AYKFGKTVVS	EGYESSEDQYVL	RGSCGLEYNLDYTEHGLKKLRE	ESGKNHGFNSH	SD <u>YY</u> KK		LYTSDSGGISC	LITIAVLLAIAFGVYKLFLSDGQDS
M F6W0G6 Equus caballus	DRILLRD	IKALTLHYDRYTTSRRLDPIPQLRC	VGGTAGCDSYTPKVIQC0	NKGWDGYDVQWECKTDLDI	AYKFGKTVVS	EGYESSEDQYVL	RG <mark>SCGL</mark> EYNLDYTELGLKKLRE	ESGKNHGFNSE	SNYYNK		LYSPDSSGMSG	LITIVVLLAIAFGVYKLFLSDGQDS
M J9NUD8 Canis lupus familiaris M M3WU58 Felis catus	GWNDP-DRILLRD	IKALTLHHDRYTTSRRLDPIPQLKC	VGGTAGCDSYTPKVIQCQ	NKGWDGYDVQWECKTDLDI	AYKFGKTMVS	EGYEFSEDQYVL	RGSCGLEYNLDYTELGLKKLRE	ESGKNHGFNSH	SNYYDK		LYSPDSSGIRG	LITILVLLAIAYGVYKLFLSDGQDS LITVIVLLAIAFGVYKLFLSDGQDS
M M3WU58 Felis catus M G1L3VO Ailuropoda melanoleuca	GWKDP-DRILLPD	VKALTLHHDRYTTSRRLDPIPOLK	VGGTAGCDSYTPKVIOCO	NKGWDGYDVOWECKTDLDI	AYKFGKTVVS	EGYESSEDOYVL	RGSCGLEYNLDYTELGLKKLRE	ESGKNHGFNSH	SDYYNK		LYSPDSSGISC	LITIVVLLAIAFGVYKLFLSDGODS
M U6DU03 Neovison vison	GWKDP-DRILLRD	VKALTLHHDRYTTSRRLDPIPQLKC	VGGTAGCDSYTPKVIQC0	NKGWDGYDVQWECKTDLDI	AYKFGKTVVS	EGYESSEDQYVL	RG <mark>SCGL</mark> EYNLDYTELGLKKLRE	ESGKNHGFSSF	?SD <u>YY</u> NK		LYSPDSSGISC	LLTIVVLLAIAFGVYKVFLSDGQDS
M G9KU82 Mustela putorius furo	GWKDP-DRILLRD	VKALTLHHDRYTTSRRLDPIPQLKC	VGGTAGCDSYTPKVIQCO	NKGWDGYDVQWECKTDLDI	AYKFGKTVVS	EGYESSEDQYIL	RGSCGLEYNLDYTELGLKKLRE	ESGKNHGFSSI	SD <u>YY</u> NK		LYSPDSSGISC	LITIVVLLAIAFGVYKVFLSDGQDS
F S4RE13 Petromyzon marinus	DRLLLRD	DVSALTLHTGKMTAG <mark>RRSAAVPQLRC</mark>	VGG <mark>C</mark> SSFTPDVVQ <mark>C</mark> LI	NRGHDGVDVQW <mark>EC</mark> KADMGS	SYRFGS ISVS	EGYDYPDDPYVL	RGSCGLEYSLVGGGGGGAGT	WR	SNNPLP		DGKGNGSG	VLIILVLIGVAFGVYKLFLSGGG
M F7C5X4 Monodelphis domestica M G3WF87 Sarcophilus harrisii	AWDQS-EKILLRD	DIQALTLHRDRFTTARRTAPIPQLQC	LGGSAGCPAHIPEIVQCR	NKGWDGFDVQWECKAELDT	SYRFGKTAVS	EGYDYPDDPYVL	RGSCGLEFNLELTEQGRKKYKI	DAGS-NANSHG-NTYG	SNYFPGY	/EKMD	SPVPSSTRC	LLAVVILLVLALGVYKLFLKAQNE- LLAVVILLVLALGVYKLFLKAQNE-
M L8Y892 Tupaia chinensis	GWHDR_GRMLLRD	VKALTLHAGRYTASPRI.DPVPOLKC	VGGTARCDSHTPTVVOCH	RGWDGYDVQWECKAELDI	AVERGETVUS	EGIDIPDDPIVL	RGSCGLEY OLDYTELGLORI.RE	ESGREPGFAS	/SDBHRR	EKMD	WYSTDSCGAGO	LLAVVILLVLALGVIKLFLKAQNE-
F V9KPW8 Callorhinchus milii	AWDGS-DRILLRE	IQALTLYNGRYTNGRRTKSVPQLKC	VGGTAGCGTFIPEVVQCY	NKGWDGYDVQWECKTDMDH	IAYRFGTIMVTC	EGYNYPEDPYVL	QG <mark>SCGL</mark> EYMIELTEEGYRKRKS	SNTG	FYSG	SPNYQ	TSYSSDSSN	VVVVLILLGVAYLLYKVFLSHQQLR
F A0A0P7VCV1 Scleropages formosus	FLSGSVLLRD	IQALTLYRGRYTTARRTSPVPQLQC	LGGSAGCNAFVPEVVQC0	NKGWDGVDVQWECKTDMDN	ITYRFGRIEVS <mark>C</mark>	EGFNHPDDPYIL	KGSCGLEYTLELTEQGRQQGYG	3SRGSGAI	FDGFAGFASSFFQGGGG	GNSRYQQGG	GAYHSSSSGGEASC	LIVVAFFLLLAYGVYKMFLCNPTH-
R H9G658 Anolis carolinensis		VQALTLYPGKNANYRRSSPVPQLQC	TGGSAG <mark>C</mark> GAYTPDVVQ <mark>C</mark> YI	NKGWDGYDVQWECKADLDA	AYRFGKIEVSC	EGYDYPDDPYIL	KGSCGLEYTLELTKAGQQKTSS	SFGGS	YYST	T	SHDSVGS	GTYQQS
R T1E3U1 Crotalus horridus R J3S9R3 Crotalus adamanteus		VQALTLYKGKHTNYRRTSPVPQLQC	TGGSAGCTAYTPEVVQCY	NKGWDGYDVQWECKAHLDV	SYRFGKIEVS	EGYDYPDDPFIL	KG <mark>SCGL</mark> EYTLELTKEGQQKANI KG <mark>SCGL</mark> EYTLELTKEGQQKANI	IFSGT	YYSA		SHDSVGSGFG	LILVILFVVFVFGIYKLFLCDNR
R AOAOB8RPT2 Boiga irregularis		VQALTLYRGKYTNYRRTSPVPQLQC		NKGWDGYDVOWECKAHLDV	SYRFGKIEVS	EGYDYPDDPFIL	KGSCGLEYTLELTAKGQQKXNI	rFSGr rFSGr	YYSA			
R A0A0F7YZ98 Micrurus fulvius		VQALTLYRGKYTNYRRTSPVPQLQC	TGGSAGCTAYTPEVVOCY	NKGWDGSDVOWECKAHLDV	SYRFGKIEVS	EGYDYPADPFIL	KGSCGLEYTLELTKEGOOKANT	rFSGT	¥¥SA	T	SHDSVGSGFG	VILVILFVVFVFGIYKLFLCDNR
R K7F509 Pelodiscus sinensis	GRVLLRE	VQVLTLYQDQYTTSRRTSPVPQLQC	TGGTAR <mark>C</mark> A-YVPAVVQ <mark>C</mark> HI	NKGWDGFDIQWE <mark>C</mark> KADLDN	ISYRFGRIEVS <mark>C</mark>	EGYDYPDDPYIL	RGS <mark>CGL</mark> QYALELTEEGQRKTNM	NFGSFG	SS <mark>YF</mark> QS	NN	KVNSQDSSDSTSC	VIVVIVLLLLAYGCYKLFLSNH
R A0A151MQ12 Alligator mississippiensis	S GWDQP-GRVLLRE	VQALTLYRDQYTTSRRTSPVLQLQC	TGGSAG <mark>C</mark> S-YVPAVVQ <mark>C</mark> HI	NKGWDGFDIQWECKADLDI	SYRFGRTEVS	EGYDYPDDPYIL	KGSCGLEYRLELTEEGERKTRM	NFG-GSSS	SNYYHS	\$	KVKPLDSSDSSTO	MIIIIVLLVLAYGVYKLFLSNL
B A0A091HXZ3 Calypte anna B A0A091QQP4 Merops nubicus	VLLRE	VQALTLHRGRYTTARRTAAVPQLQC VQALTLHKGQYTASRRTAAVPQLQC	TGGTAGCS-RVPEVVQCY	NKGWDGYDVQWQCKAELEN	ISORFGOMEVSO	EGYDHPDDPYIT	RGSCSLLFSLELTEEGERRM-N	NSNRFH	SGNYHL	K	DDYSSCGVG	ALVLLVL-AVVYGIYSYFHGKYSSG
B H0Z2U4 Taeniopygia guttata	VLLRD	VQVLTLHRGQYTTSRRTASIPQLQC	TGGSAGCS-HVPEVVOCY	NKGWDGYDVOWOCKADLEN	TYRFGOMEVSC	EGYDYPDDPYIL	RGSCSLLFKLELTAEGERKMKN	NSGSFG	SSYYOS	RR	KDYSDFGSG	AIVVIVLIVLAFGVYKFFLSS-0
B A0A091ES15 Corvus brachyrhynch	VLLRD	VOVLTFHRGOYTTSRRTAALPOLOC	TGGSAGCS-RVPEVVOCY	NKGWDGYDVOWOCKADLEN	AYRFGOLEVS	EGYDYPDDPYIL	RGSCSLLFRLELTEEGERKVKN	NSGSFG	ssyyos	RR	KDYSDSGSG	AIVVIVLLILAFGVYKFFLSNNO
B U3JW28 Ficedula albicollis	GWDRP-GKLLLRD	VQALTLRRGQYTTSRRTAAVPQLQC	TGGSAG <mark>C</mark> S-RVPEVVQ <mark>C</mark> YI	NKGWDGYDVQWQCRADLEN	IAYRFGQMEVSC	EGYDYPDDPYIL	RGSCSLLFNLELTEEGERKVKN	NSGSFG	SSYYQL	RR	KDYSDSGSG	AIVVIVLLVLAFGVYKLFLSN-Q
B A0A091MMQ1 Acanthisitta chloris B A0A094NHX5 Antrostomus carolinensis	VLLRE	VQALTLRRGQYTTARRTAALPQLQC	TGGSAGCA-HVPEVVQCY	NKGWDGYDVQWQCKADLDN	AYRFGQIEVS	EGYDYPDDPYIL	RGSCSLLFSLELTKEGERKVK	NSGSFG	SNYYQP	W	KDSSDCGSC	AIVVVVLLILAFGVYKLFLSN-Q
B A0A093I3S6 Struthio camelus au	VLLRE	VOALTLERGOYTTSRETAAVPOLOC	TGGTAGCS-PVPEVVOCY	RGWDGYDVOWOCKADLEN	ISYRFGOIEVSC	EGYDYPDDPYIL	RGSCGLLFRLELTEEGERKAKN	NSGSFG	SDSY05	R	KDFSDSGFG	VIVVIVLLILAYGVYKLFLSN-O
B U3IP68 Anas platyrhynchos	DRVLLRE	VQALTLRRGQYTTSRRTAAVPQLQC	TGGSAGCS-RAPEVVQCY	NKGWDGYDVQWQCKADLEN	THRFGQIEVS	EGYDYPDDPYIL	RG <mark>SCSL</mark> LFRLELTEEGERKVKN	NSDSFG	FYQS	RR	KDSSDSGAG	AIVIIVILVLAYGVYKFFLSN-Q
B A0A094LXF6 Podiceps cristatus	VLLRE	VQALTLHRGQYTTSRRTAAVPQLQC	TGGTAGCS-RVPEVVQCY	NKGWDGYDVQWQCKADLEN	IIYRFGRIEVS <mark>O</mark>	EGYDYPDDPYVL	RGSCSLLFRLELTEEGERKVKM	NSGSFG	SG <mark>YY</mark> QS	RR	KDSSDAGSG	AILIIVLLILAFGVYKLFLGN-Q
B A0A093PUG3 Manacus vitellinus	VLLRD	IQALTLHRGQYTTSRRTPAVPQLQC	TGGTAGCS-RVPEVVQCY	NKGWDGYDVQWQCKADLDF	CAYRFGHIEVS C	EGYDYPDDPYIL	RGSCSLLFRLELTEEGERKVK1	rs	ssyygs	R	HDSSDSGSG	AIVVIVLLALAFGVYKFFLSN-Q
B AOA087VMM4 Balearica regulorum B AOA091RLX7 Mesitornis unicolor	VLLRE	VOALTLEVGOTTTSRRTAAVPOLOC	TGGTAGCS-RVPQVVQCY	KGWDGYDVQWQCKADLEN	THREGRIEVS	EGYDYPDDPYIL	RGSCSLLFTLELTEEGERKVKN	NSGSFG	SGYYQS	RR	KDSFDSSAC	AIVIIVLLVLAFGVYKLFLCN-Q VIVVIVLLGLAFGVYKLFLSN-Q
B A0A093FU90 Picoides pubescens	LLLRE	VQALTLHRGQYTTSRRTPAVPOLOC	TGGTAGCS-HVPEVVOCY	NKGWDGYDVOWOCKADLER	TYRFGOIEVSC	EGYDYPDDPYIL	RGSCSLLFRLELTEEGERKVKM	NSGSFG	SSYY0S	RR	KDSSDSVG0	AIIIIVLLVLAFGVYKLFLSN-E
B A0A091G8A3 Cuculus canorus	VLLRE	VQALTLHRGLYTASRRTAAVPQLQC	TGGTAGCS-HVPEVVQCY	NKGWDGYDVOWOCKADLEN	IIYRFGOIEVSC	EGYDYPDDPYIL	RGSCSLLFRLELTEEGERKVKN	NSGSFD	YGFYOS	RR	KDSSDSSAG	AIVIIVLLVLAFGVYKLFLSN-0
B AOA091LP00 Chlamydotis macqueeii	VLLRE	VQALTLHRGQYTTSRRTAAVPQLQC	TGGTAGCS-RVPEVVQCY	NKGWDGYDVQWQCKADLEN	ITYRFGQIEVSC	EGYDYPDDPYIL	RGSCSLMFRLELTEEGERKVKN	NSGSFG	SGYYQS	RR	KDSSDSGAG	AIVIIVLLALAFGVYKLFLSN-E
B A0A091IV81 Egretta garzetta B A0A091HD25 Buceros rhinoceros	VLLRE	VQALTLHRGQYTTSRRTAAVPQLQC	TGGTAGCS-RIPEVVQCY	NKGWDGYDVQWQCKADLEH	ITYRFGQIEVSC	EGYDYPDDPYVL	RGSCSLIFRLELTEEGERKVKN	NSGSFG	SGYHQS	R	KDPSDSGAG	ALVIIVLLVLAFGVYKLFLSN-Q
B A0A091HD25 Buceros rhinoceros B A0A0A0B005 Charadrius vociferus	ILLRE	VQALTLHRGQYTTSRRTAAVPQLQC VQALTLHRGQYTTSRRTAAVPQLQC	TGGTAGCS-HVPEVVQCY	KGWDGIDVQWQCKADLEI	TYREGRIEVSC	EGYDYPDDPYIL	RGSCSLMFRLELTEEGERKVKN	NSGSFG	ssirgs	K R	KDSSDSGAG	ATVIIVLLVLAFGVIKLFLSN-Q
B A0A091TB48 Phaethon lepturus	VLLRE	VQALTLHRGQYTTSRRTAAVPQLQC	TGGTAGCS-RVPEVVQCY	NKGWDGYDVQWQCKADLEN	TYRFGQIEVS	EGYDYPEDPYIL	RGSCSLLFRLELTEEGERKVKN	NTGSFG	sgyyos	R	KDSSDSGAG	AIVIIVLLILIFGVYKLFLGN-Q
B A0A093T3W7 Phalacrocorax carbo	ILLRE	VOALTLERGOYTTARE TAAVPOLOC	TGGSAGCS-RVPEVVOCY	NKGWDGYDVOWOCKADLEN	AYRFGOIEVS	EGYDYPDDPYIL	RGSCSLLFRLELTEEGERKVKN	NSGSFG	SGYY0S	RR	KDSPDSGAG	AIVVIVLLVLAFGVYKLFLGN-O
B A0A09102D7 Leptosomus discolor	VLLRE	VQALTLHRGRYTTSRRTAAVPQLQC	TGGAAGCS-RVPEVVQCY	NKGWDGYDVQWQCKADLEN	AYRFGQIEVS	EGYDYPDDPYIL	RGSCSLLFRLELTEEGERKVKN	NSGSFG	SSYYQP	RR	KDSSDSGAC	AIVIIVLLALAFGVYKLFLSN-Q
B AOAO87R9R1 Aptenodytes forsteri B AOAO93PAE4 Pygoscelis adeliae		VQALTLHRGQYTTSRRTAAVPQLQC										
B AOA093PAE4 Pygoscells adellae B AOA091SJK4 Pelecanus crispus		VQALTLARGQYTTSRRTAAVPQLQC										
B A0A091W0S0 Nipponia nippon	VLLRE	VQALTLRRGQYTTSRRTAAVPQLQC	TGGTAG <mark>C</mark> S-RVPEVVQ <mark>C</mark> Y	NKGWDGYDVQWQCKADLEN	TYRFGQIEVS <mark>C</mark>	EGYDYPDDPYIL	RGS <mark>CSL</mark> LFRLELTEEGERKVKM	NSGSFG	sg <mark>yy</mark> qs	RR	KDPSDSGAG	AIVIIVLLALAFGVYKLFLSN-Q
B G1N8U4 Meleagris gallopavo		VQVLTLHRGRYTTARRTAAVPQLQC										AIFVIVLLILAFGVYKFFLSN-Q
B F1P4D5 Gallus gallus A F7C1E2 Xenopus tropicalis	GWEQQ-G-VLLRD	VQVLTLHRGRYTTARRTAAVPQLQC	IGGSAGCS-DIPEVVQCY	NRGWDGYDVQWQCKADLEN	TLRFGQIEVSC	EGYNYPDDPYIL	RG <mark>SCSL</mark> LFRLELTKEGEWKAKN RG <mark>SCGL</mark> EYTLELTEEGRKRSQI	NSGGFG	VRCCECCECCURAS	T	KDSSDSGAG	TIFVIVLLILAFGVYKFFLSN-Q
A F7C1E2 Xenopus tropicalis A Q0IHF9 Xenopus laevis	CWNNQ-ERVLERD CWSPO-DRVLLRD	DIQAITLYADRYTNARRSAPVPQLKC	I GGNAGCHAMVPQVVQCH	NRGWDGLDVOWECRVDMDN	ISTREGEVEVSC	EGEDEPEDPIVL	RGSCGLEYTLELTERGERETOR	DG		NSOSWD	SKTDGSS	AIVLIVIVIIAYGVYKLFLSGPSV- AIVLIFIVILAYGVYKLFLSGPSV-
F W5U934 Ictalurus punctatus	CWNDGAVLLRD	VQALTLYRGRYTTARRSSPVPQLQC	VGGSAGCTAFVPEVVQCQ	NKGWDG <mark>VDVQWEC</mark> KADMDN	WYRFGKVEVS	EGYNNPDDPYIL	RGSCGLEYTLELTAEGRQN	QGS	FRFSDFASG <mark>FF</mark> QG	KQQHGS	SQQFQSGLSAEGSGS	LVVIALFLLLAFAVYKMFLCDSGR-
F F1QPC3 Danio rerio		VQVLTLYRGRYTTARRSSPVPQLQC	IGGSAGCGSFTPEVVQCY	NRGSDGI DAQWECKADMDN	ILYRFGRVEVS	EGYNSPNDAYVL	RGSCGLEYTLELTAEGKQQ	QGS	SGFSGFASNFFQGFSN	QKQQQRGGGGDG	GG-PYQQHSYQNSPSGDGVGC	LLVVAFLLLVAFVVYKMFLCSPTN-
F H3A654 Latimeria chalumnae	GWNSH-ESVLLQE	VQVLTLYKNRFTTARRTSPVPQLQC	NGGTAG <mark>C</mark> SAFVSEVVQ <mark>C</mark> QI	NKGWDGFDVQWECKTDMDN	INYRFGTIQ <mark>VN</mark> C	EGYNSPDDPYVL	KG <mark>SCGL</mark> EYTLELTEAGRMKRNF	KGYGSGG	FSSNFFKD	FSNNVN	SPVSSDSSG	VLVVVILLVIAYGIYKIFLSSPQS-
F W5M444 Lepisosteus oculatus F H2SFE8 Takifugu rubripes	SWNDG-GSVLLRE	LQVITLYKGQYTNSRRTSPVPQLQC	IGGTAGCGSFIPEVVQCH	NKGWDGFDVQWECKTDMDY	SYRFGKIEVS	EGYNYPDDPYVL	KGSCGLEYTLELTEEGKQKGRM	NS	RSFGSGFFQ	DSSSNVNYQ	OWNERS CONCEASED	LMVVAVLLLLAYGVYKMFLCGPHG- LLVVAVLLLMAYGVYKLFLSENI
F H2SFE8 Takifugu rubripes F H3DKA5 Tetraodon nigroviridis	SLHGDAVLLOD	VQVLTLYRGLYTTARRSSPVPQLQC	VGGSAGCOAFVPEVVQCQ	NRGWDGVDVQWECKTDMDN	ATREGETEVSC	EGYDHPSDAVIL	KGSCGLEYNLELTEEGPRENAG	shsG 3spra	FNAFEGSG	rGSSGDNRKP	SHHSSDSSDVG_FSN	LLVVAGLLLVAYCFYTFA
F B5X2Y8 Salmo salar	SWNEAPGSVLLRD	VOVLTLYKGKYTTARRSSAVPOLOC	VGGSAGCGSFIPEVVOCK	NKGWDGVDAOWECKTDMDN	AYKFGRIEVS	EGFNHPDDSYIL	KGSCGLEYSLELTEEGKRRHKG	GSHGSTGGFS-	DFASSFFKG	SSDNNNOHHH	S-NNROOGSTGEGNSSO	LVVVALLLFLAYGVYKLFLSGPTN-
F A0A060VS03 Oncorhynchus mykiss	SWNEGOCSVLLRD	VOVLTLYKGKYTTARRSSAVPOLOC	VGGSAGCGSFIPEVVOCK	NKGWDGVDAOWECKTDMDN	AYKFGRIEVS	EGFSHPDDAYIL	KGSCGLEYSLELTEEGKRRHKG	GSHGSTGGFS-	DFASSFFKG	SSDNNNOHHH	S-NNROOSSTGEGNSSG	LVVVALLLFLAYGVYKLFLSGPTN-
F G3PVR5 Gasterosteus aculeatus	SWDDGSVLLRD	DLQALTLYKNRYTTARRASPVPQLQC	VGGSAG <mark>C</mark> QAFVPEVVQ <mark>C</mark> QI	NKGWDGVDVQWECRTDMDN	AYRFGRIEVS	EGYSHPADALIL	KGS <mark>CGL</mark> EYTLELTEHGRSTSHO	3G	TKSFGGR	R	IDDAKSR	LLVVAVLLLVAYGVYKLFLSGEPR-
F I3KGM8 Oreochromis niloticus F AOA146NC37 Fundulus heteroclitus		IQALTLYRNRYTTARRSSPIPQLKC	VGGSAGCHAFVPEVVQCVI	NKGWNGFDVQWECRTDMDN	TYREGHIEVSC	EGYSHPADAVIL	KGSCGLEYTLELTEEGQRRAHS	SSRGSRGGFKI	DL-WGFASSFFSG	SSEYGQQ		LLVVAVLLLLAYGFHKLFLSGNN LLVVAVLLALAYGVYKLLLSGDT
F A0A146NC37 Fundulus heteroclitus F A0A087XZZ6 Poecilia formosa			VGGSAGCHVFIPDVVOCVI	NTGWDGVDVOWECKTDMDN	AYRFGAIEVSC	EGYSHPADDYIL	KGSCGLEFTLELTOEGKRKTLS	Sar Ganger Ki		NKRH	OHYAODGESSGC	LLGVAVLLALAYGYYKLLLSGDT
F M4ANEO Xiphophorus maculatus	SWNQDSVLLRD	IQALTFYRNRFTTARRSNPVPQLKC	VGGSAGCHVFIPDVVQCVI	NRGWDGVDVQWECKTDMDN	AYRFGAIEVS	EGYSHPSDDYIL	KGSCGLEFTLELTQEGQRRTQC	3SFGSQGGFKQ	W-GGLGGLASSFFSSFFG	NRHHQHQQHQ	Q-QHQQQHYAQDGESSGC	LLVVAVLLLIAYGLYKLFLSGDT

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Vertebrate class: A, Amphibian; B,Bird; F,Fish; M,Mammal; R, Reptile

26 Feb 19

Fig. S2 SARAF_L **vertebrate homologs**. Sequence comparison of Human SARAF_L and vertebrate homologs. Disulfides, SS1, SS2, and SS3 are indicated. Conserved cysteines (yellow), invariant residues (blue), and conserved residues (green) are highlighted. Human SARAF_L secondary structural elements and transmembrane domain, TM (grey) are indicated. Vertebrate class is indicated as A, amphibian; B, bird; F, fish; M, mammal; and R, reptile.

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# 000432 Aspergillus terrous YRAFERPEGKNAILLSKV0TLITAA-RLFSSRVS FIPULKCV0PSKI ICDXYFIPDMCTAGLPEFFLGATDVVCEGYRNDDKVLKGSCGVEYRLLLFERGESRGRIQEDDMSTPLATKGWRAGNDALVULFIPGFVACFLIMHYPHLWEQCG # B0YD10 Messartorya fumigatus YGRSSESPPGPNAILLSKV0STLTAA-RLFSSRVSFIPOLKCV0PSKI												
B 00101 Nossartorya fumigata UGRSESPPGPNAILLSRV0STIPAA-RLTSSRV91PDLKQVGPSKTIDKYNIETMKCTNAGTYDDEEDVQMTCTAQLPEEPHLGADVICEGURNADDKWLKGSGGVEYKLLLERGESRFGRIQEDDMRSIPLATKGWRRGMDAUDULFLGFNAAUVUNIFWPMVRSGG H 04WDD Asporgillus funigata UGRSESPPGPNAILLSRV0STIPAA-RLTSSRV95 IPDLKQVGPSKTIDKYNIETMKCTNAGTYDDEEDVQMTCTAQLPEEPHLGADVICEGURNADDKWLKGSGGVEYKLLLERGESRFGRIQEDDMRSIPLATKGWRRGMDAUDULFLGFNAAUVUNIFWPMVRSGG H ALDLS9 Nossartorya fischori UGRSESPPGPNAILLSRV0STIPAA-RLTSSRV95 IPDLKQVGPSKTIDKYNIETMKCTNAGTYDDEEDVQMTCTAPLPEEPHLGADVICGGVENADDKWLKGSGGVEYKLLLERGESRFGRIQEDDMRSIPLATKGWRRGMDAUDLIFLGFNAAUVUNIFWPMVRSGG H ALDLS9 Nossartorya fumigata FLSSKV95 IPDLKQVGPSKTIDKYNIETMKCTNG4DYDEEDVQMTCTAPLPEEPHLGADVICGGVENADDKWLKGSGGVEYKLLLERGESRFGRIQEDDMRSIPLATKGWRRGMDAUDLIFLGFNAAUVUNIFWPMVRSGG H 61KN3 Asporgillus clavatus FGRSSSSPARNAILLSRV0STIPAA-RLTSSRV95 IPDLKQVGPSKRCUNXIFDTMCTNG4DYDVEDUQWTCTAPLPEEFHLGADVICGVENADDKWLKGSGVEYKLLLERGESRFGGLGADKUMSTSSSSSEWRRGMDAUDLIFLGFNAAUVUNIFWPMVRSGG G 7XB96 Asporgillus kawachii FSPSKNAXILSRV0STIPAA-RLTSSRV95 IPDLKCVGPSKRCUNXIFDTMCTNG4DYDVEDUQWTCTASLPOEFLLGADVICGVENADDKWLKGSGVEYKLLLERGEERFGHLRMESSSSSDELGPWRRGMDAUDLIFLGFNAAUVUNIFWMVRSGG G 7XB96 Asporgillus kawachii FSPSKNAXILSRV0STIPAA-RLTSSRV95 IPDLKCVGPSKRCUNYIFDTMCTNG4DYDVEEDVQMTCTASLPEEFHLGADVICGGVEYKLLLERGEERFGHLRMESSSSDELGP												
# 04MD9 Aspergillus fumigatus YGRSESSPEGNAILLSRVOSLTLRAN -RLTSSRRVSPIPDLKOVGFSKTIDDXYNIETMACTNAUTVIEEMVONTCTAUPEEFHLGATDVICEOYENADDXWULKGSCGVEYRLLLERGERRGCIDDMRSIPLATKGWRRGHDALUDLIFLGFNAAUUVINIFWPWNSCGG # AlDLS9 Moosartory fischori YGRSESSPEGNAILLSRVOSLTLRAN -RLTSSRRVSPIPDLKOVGFSKTIDDXYNIETMACTNAUTVIDEEDVOWTCTAPLPEEFHLGATDVICEGYRNADDXWULKGSCGVEYRLLLEEGERRGCIDDMRSIPLATKGWRRGHDALUDLIFLGFNAAUUVINIFWPWNSCGG # AlDLS9 Moosartory fischori YGRSESSPEGNAILLSRVOSLTLRAN -RLTSSRRVSPIPDLKOVGFSKTIDDXYNIETMACTNAUTVDEEDVOWTCTAPLPEEFHLGATDVICEGYRNADDXWULKGSCGVEYRLLLEEGERRGCIDEEDMSOFSSIG												
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H AlCM87 Aspergillus clavatus FGRSSESSPARNAILLSRV0SLTLRAM-RLTSSRRVSPIPOLKCVGPSKRCLUYIDTMKCTVAGHDYDEEDUVGTCTAPLPPEFKLGSTDVVCGYRNADDKWVLKGSCGVEYRLLLELGERRFGTEEDLMSGFSSTKGWRGMSVLGDLIFFGPMGAVFILINPMVRDGFG B6HDMO Penicillium rubens FSNSKPPGKDAILLSNV0SLTLRAM-RLTSSRRVSPIPOLKCVGPSKRCNNISPETMCTN0GYDDVEDUVGYCTADLPPEFKLGATDVVCGGVRNADDKWVLKGSCGVEYRLLLEAGEKRFGRHRRSSSSSREGFGVGKUIFFGFMIAVFPHILMAFFNECCG A2R4M9 Aspergillus niger												
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P 08438 Schizophyllum commune												
P B3LB94 Puccinia graminis sp. tritici NVSNSKVLSEIPTLTFFDG-EKTNFRRTSPVDLVCQGFGCNIFRPDVIGCYNKGGSGTDVNWKCEADLPAKLKLGRVEVGCEGNSNANDPHVLKGSCALTYTLKFDASSKYDSSHNGGLT		MARDRILLE	IPALTFYAG-EDTV	ARRSDPIPOLTCIGKP	- <mark>C</mark> KIYQPEVVR <mark>C</mark> TNTGGTG	TEVEWKCKA	DLPEALRFGRVEVS	SCEGYSRPGDPYVLKGS	CGLQYKLVQ	PGALRNEDEGWADS-	WNTFSECAYI	HLYPFLFFSSLTFRPYSHRRPHHSL
P P4R312 Melampsora larici populina MATQKRVLLSSIDTLTFYDG-ERTHFRRTSPVLDLVCQGQPCKRFKPDVICCQNTGGSGSNIHNKCEADLPSSIKLGRTEVSCEGFRNADDPYILEGSCGLTYTLKDAGFSSHTRDGMSLEGFLFWALFAIVGIFIIYPLRSLFSTALRPVSRYPG P 678927 Mixia osmundae MTTGSKVLLSSIDTLTFYDG-ERTHFRRTSPVDLTCQGSKSICKRFKPDVICCQNMGGTAPDIQNKCEAELPSSVKLGKPVSCEGAAHADDFYILGSCGLTYTLKDAGFSSHTRDGMSLE		MVSNSKVLLSE	IPTLTFFDG-EKTN	IFRRTSPVDQLVCQGPG	- <mark>C</mark> NIFRPDVIQ <mark>C</mark> YNKGGSG	TDVNWKCEA	DLPAKLKLGRVEVO	G <mark>CEGWSNAND</mark> PHVLKGS	CALTYTLKFI	ASSKYDSSHNDGLT-	GQCLPSEAEH	S
E62VX0 Sporisorium reilianum YTPNAQYYGAGRRVAMSSIETLTFYAG-RRTAFRRTSALPQLTCVGSACARYQPDVVQCTAMGDAQMXCSADLPRTMRLGRVEVSCEGYDYAQDPYVLKDSCALEYHLLPSGTGPDDPWRTYQRGAWGDKVLDALFQLVFWSVLAVIVVSFVRAIRGG		MATQKRVLLS	IDTLTFYDG-ERTN	IFRRTSPVLQLVCQGQP	- <mark>C</mark> KRFKPDVIQ <mark>C</mark> QNTGGSG	SNIHWKCEA	DLPSSIKLGRTEVS	S <mark>CEGFRNADDPYILEGS</mark>	CGLTYTLKD	AGFSSSHTRDGMSLE-	GFLFWALFAI	VGIFIIYPLLRSLFSTALRPVSRYFPG
	P G7E9Z7 Mixia osmundae											
	E6ZVXO Sporisorium reilianum			FRRTSALPQLTCVGS	M <mark>C</mark> ARYQPDVV <mark>QC</mark> TAMG	DAQWK <mark>C</mark> SA				GTGPDDPWRTYQRG-	AWGDKV	LDALFQLVFWSVLAVIVVSFVRAIRGG
		:.*:	: :**: *.	** . ** *	* * * *	::* *	: *.	. <mark>*</mark> **: * ::*::*	*::			

Pathogen: A, animal; H, human; I, insect; P, plant

Fig. S3 SARAF fungal homologs Sequence comparison of Human SARAFL and fungal homologs. Disulfides, SS1, SS2, and SS3 are indicated. Conserved cysteines (yellow), invariant residues (blue), and conserved residues (green) are highlighted. Human SARAFL secondary structural elements and transmembrane domain, TM (grey) are indicated. Host for pathogenic fungi is indicated as A, animal; H, human; I, insect; and P, plant.

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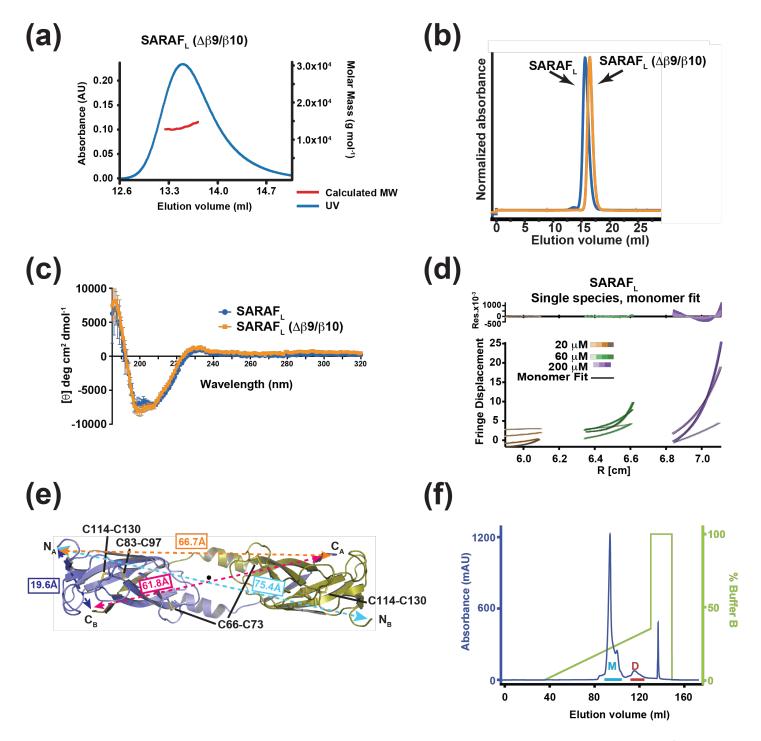


Fig. S4 Characterization of engineered SARAF_L mutants (a) SEC-MALS of 72.5 μ M (1 mg ml⁻¹) SARAF_L ($\Delta\beta9/\beta10$) (experimental Mw of 13.96 kDa ± 0.10, Mw/Mn = 1.002 ± 0.010, predicted monomer Mw of 13.78 kDa). (b) SEC of SARAF_L and SARAF_L ($\Delta\beta9/\Delta\beta10$) run at ~1 mg ml⁻¹and 3 mg ml⁻¹, respectively. (c) Circular dichroism spectra of 10 μ M SARAF_L (orange) and 10 μ M SARAF_L ($\Delta\beta9/\beta10$) (blue) at 4°C in 30mM sodium phosphate pH 7.4. (d) Single species fit for equilibrium ultracentrifugation of SARAF_L at the indicated

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concentrations. Rotor speeds of 10K, 18K, 22K and 31K rpm are denoted by increasingly darker shades for each concentration. Residuals show fits to a single species model. **(e)** SARAF_L dimer views from the putative membrane facing side. Distances between the N and C termini from the A and B subunits are shown. N_A - N_B (cyan), 75.4 Å; N_A - C_A (orange), 66.7 Å; N_A - C_B (dark blue), 19.6 Å; C_A - C_B (magenta), 61.8 Å. **(f)** Ion exchange (MonoQ) chromatogram showing separation of monomeric (M) and dimeric (D) forms for disulfide-locked SARAF_L.

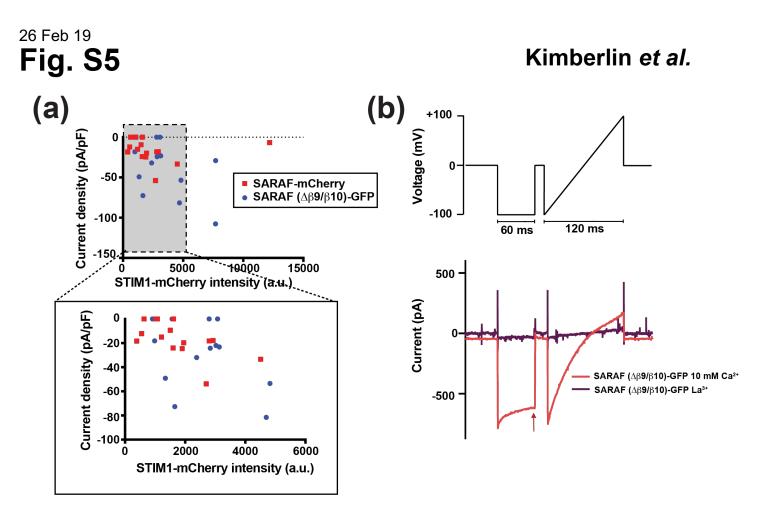


Fig. S5 SOCE current amplitude is positively correlated with STIM1 expression levels (a) Comparison of current density and STIM1 expression levels. Fluorescence of each cell was measured before recording in two channels for assessing the levels of STIM1-mCherry and SARAF-GFP expression. Peak current density (maximal current density reached after activation) of each cell was plotted against STIM1-mCherry fluorescence levels. General tendency of cells to express higher SOCE density when higher levels of STIM1-mCherry is expressed as a positive correlation between the two parameters as seen on the graph. Inset shows a zoomed in view for cells having STIM1-mCherry fluorescence intensities \leq 5000 a.u. (b) Recording protocol (top) with exemplar traces (bottom). Cells were held at 0 mV followed by a 60 ms a step to -100 mV, a 20 ms return to 0 mV, and ramp from -100 mV to +100 mV. The command was delivered at 0.5 Hz. Red arrow indicates the time point at the steady state where measurements were taken for further analysis. To subtract leak from each current value, lanthanum is applied each time after the protocol (purple).

Supplementary Table S1 Crystallographic data collection and refinement statistics

	SARAF∟ Native		SARAF∟ K₂Pt(NO)₄		SARAF _{L.SS} Monomer	SARAF _{L.ss} Dimer
Data Collection						
Wavelength (Å)	1.1271	1.0432	1.0702	1.0705	1.1159	1.1159
		(Remote)	(Peak)	(Inflection)		
Space group	C2		C2		P212121	P1
Cell dimensions a/b/c (Å)	50.98, 63.93, 79.92		52.08, 63.92, 78.53		59.69, 60.95, 63.44	24.91, 30.79, 76.14
α, β, γ (°)	90.00, 100.70, 90.00		90, 99.59, 90		90.00, 90.00, 90.00	88.58, 86.03, 89.79
Resolution (Å)	39.43 - 1.80	77	.38 - 2.16 (2.28-2.16)		43.95 - 1.58	37.93 - 2.10
	(1.84-1.80)				(1.64 - 1.58)	(2.18 - 2.10)
R _{pim} (%)	2.8 (84.6)	3.5 (38.1)	3.3 (34.9)	3.5 (44.1)	3.8 (30.5)	4.6 (56.1)
Mn (I/σI)	12.1 (0.7)	13.4 (2.0)	13.8 (2.1)	13.6 (1.9)	16.2 (2.5)	12.3 (1.3)
CC _{1/2}	0.999 (0.349)	0.996 (0.728)	0.996 (0.777)	0.997 (0.670)	0.999 (0.781)	0.997 (0.609))
Completeness (%)	98.2 (83.7)	99.4 (97.8)	98.2 (89.8)	98.2 (89.9)	98.7 (91.5)	85.1 (43.9)
Redundancy	6.9 (5.4)	6.3 (6.1)	6.2 (5.7)	6.2 (537)	6.6 (4.3)	3.7 (3.8)
Unique reflections	23024 (1167)	13624 (1931)	13458 (1771)	13460 (1772)	32028 (4214)	11188 (461)
Refinement		•				
Rwork / Rfree (%)	19.34 (40.43) /				16.54 (21.69) /	17.46 (30.46) /
	22.75 (46.35)				20.31 (24.42)	23.36 (33.99)
Resolution (Å)	37.50 - 1.80				43.95- 1.58	30.78 - 2.10
	(1.86-1.80)				(1.64 - 1.58)	(2.18 - 2.10)
No. of chains in AU	2				2	2
No. of protein atoms	1938				2176	1994
No. of ligand atoms	n/a				9	6
No. of water molecules	152				320	89
RMSD bond lengths (Å)	0.007				0.006	0.007
RMSD angles (°)	0.93				0.85	0.93
Ramachandran best/disallowed (%)	98/0				98/0	96/0

*Values in parentheses are for highest-resolution shell.