Supplementary Information

Construction of plasmids

pStrepHA-SGFP2

pStrepHA-SGFP2 (shortened name for pEXPR-IBA105-C/3xHA-SGG-SGFP2-N1) was constructed by multi-step manipulation of modifying mammalian epitope tag vectors (FLAG, Strep, HA, and SGFP2). First, a Strep vector pEXPR-IBA105-C was constructed by inserting an oligonucleotide block (5'-gaattcggggatccgatatctcgaggtacca-3'; 5'-agcttggtacctcgagatatcggatccccgaattcgc-3') at the SacII/HindIII site of pEXPR-IBA105 (IBA GmbH, Göttingen, Germany) to generate the multiple cloning sites with a different reading frame. Next, pCMV-3xHA-A was constructed by digesting pCMV-3xFLAG-A [Suppl. Ref 1] with NotI and BamHI to remove a 3xFLAG-encoding DNA fragment and inserting a pair of 2xHA-encoding oligonucleotides (5'-ggccgccaccatggtttacccatacgatgttcctgactatgctggctatccctatgacgtcccggactatgcag-3'; 5'-gatcctgcatagtccgggacgtcatagggatagccagcatagtcaggaacatcgtatgggtaaaccatggtggc-3') and then by further inserting a pair of 1xHA-encoding oligonucleotides (5'-gatcttatccatatgacgttccagattacgctccgg-3'; 5'-gatcccggagcgtaatctggaacgtcatatggataa-3') at the BamHI/BglII site. Human NFAT2 cDNA clones (FLJ76687, encoding 1-716 a.a. of UniProt ID O95644-2, NFATc1 Isoform A-alpha; FLJ61216, encoding 473-943 a.a. of UniProt ID O95644-1, NFATc1 Isoform C-alpha) were obtained from NITE Biological Resource Center (NBRC) and used as template DNAs to amplify an NFAT2 cDNA encoding 1-943 a.a. by the overlap PCR method to fuse 1-503 a.a. and 495-943 a.a. using two pairs of primers (Pair 1, forward, 5'-ttacgctccgggatccccaagcaccagctttccagt-3', reverse, 5'-tggacacggtcttccctgtgatgcggtg-3'; Pair 2, forward, 5'-caccgcatcacagggaagaccgtgtcca-3', reverse, 5'-cggtatcgataagcttctaggagtgggtgctcgtgc-3'; overlapped primers encoding 495-503 a.a. underlined) and inserted into the BamHI/HindIII site of pCMV-3xHA-A with an In-Fusion® HD Cloning Kit (Clontech/TakaraBio). The resultant plasmid pCMV-3xHA-A/NFAT2 (1-943) was used for construction of pStrepHA-NFAT2 by inserting a PCR-amplified DNA fragment (primers: 5'-ccgagaccgcgaattcgaccatggtttacccatacgat-3'; 5'-ctgcggccgcaagcttcgaggtcgacggtatcgata-3') into the EcoRI/HindIII site of the above described pEXPR-IBA105-C. The resultant pStrepHA-NFAT2(1-943) was digested with BamHI and HindIII to remove the NFAT2 cDNA fragment, followed by insertion of a cDNA fragment encoding SGFP2 using pSGG-SGFP2-N1 [Suppl. Ref 2] as a template and a pair of primers (forward, 5'-ttacgctccgggatctgagctcaagcttcgaattctgc-3'; reverse, 5'-

ctgcggccgcaagctacgctttacttgtacagctcgtc-3') in which the HindIII site downstream of the SGFP2-encoding DNA segment was destructed.

pSP-StrepHA-SARAF

To construct pSP-StrepHA-SARAF, a synthetic DNA block encoding the SARAF signal peptide sequence (1-MAAACGPGAAGYCLLLGLHLFLLTAGPALG-30) was first inserted between the XbaI and NheI sites of pStrepHA-SGFP2 using the following two pairs of partially complementary oligonucleotides: SigPep-F1, 5'-ctagacccaccatggctgcagcatgcggacctggagcagcaggctactgcttgctcctgg-3', SigPep-F2, 5'-gactgcatctgttcctgctgacagctggacctgcactgggtg-3'; SigPep-R1, 5'-ctgctgctccaggtccgcatgctgcagccatggtgggt-3', SigPep-R2, 5'-ctagcacccagtgcaggtccagctgtcagcaggaacagatgcagtcccaggagcaagcagtagc-3' (complementary sequence in the primers SigPep-F1 and SigPep-R2 underlined; SigPep-F1 and SigPep-R2 phosphorylated by the T4 kinase reaction *in vitro*). The resultant plasmid pSP-StrepHA-SGFP2 (shortened name for pEXPR-IBA105-C/SARAF(1-30) 3xHA-SGG-SGFP2) enabled secretion of StrepHA-SGFP2 into the culture medium from transfected HEK293T cells. For replacement of the SGFP2-encoding DNA segment of pSP-StrepHA-SGFP2 with the DNA fragment encoding SARAF 31-339 a.a., In-Fusion PCR was performed with a pair of primers (forward, 5'-atctgagctcaagctttggaacgaccctgacagaatg-3'; reverse, 5'-gcggtttaaacttaattatcgtctcctggtaccacc-3', complementary sequence of re-created stop codon underlined) using pSGG-SGFP2-N1/SARAF as a template, and the amplified DNA was subcloned into the HindIII/AflII site of pSP-StrepHA-SGFP2, in which the HindIII site and the AflII site were located upstream and downstream of the SGFP2-encoding DNA segment. The mature protein encoded by pSP-StrepHA-SARAF is designated StrepHA-SARAF in this article.

pSP-HiBiT-3xHA-SARAF

HiBit-3xHA- and TwinStrep-tagging vector for single-pass transmembrane (TM) proteins (pCDNA3.1-SP-HiBiT-HA_TM_TwinStrepKR; sequences for signal peptide and TM from SARAF) was prepared by a gene synthesis service at GenScript (Piscataway, NJ, USA). A SARAF cDNA fragment without a stop codon was inserted between the BamHI site (located upstream of the TM region) and the EcoRV site (located between TM and TwinStrepKR) to construct an expression plasmid for SP-HiBiT-3xHA-SARAF-TwinStrep that was designed for assays combining Strep-pulldown and HiBiT detection.

A stop codon was re-introduced to express C-terminally untagged SARAF (SP-HiBiT-HA-SARAF). The mature protein name encoded by pSP-HiBiT-3xHA-SARAF is designated HiBiTHA-SARAF in this article.

pCX4-hygro

A retrovirus expression vector containing the hygromycin resistance (*hygro*) gene was constructed by replacing the blasticidin resistance (*bsr*) gene of pCX4-bsr [Suppl. Ref 3] with the *hygro* gene in pNL(NlucP/CRE/Hygro) (Promega) as briefly described below. pCX4-bsr was digested with NcoI and SalI to remove the *bsr* gene, and the remaining large vector fragment was isolated and ligated with the *hygro* gene by the Infusion-PCR method using a pair of specific primers (forward, 5'-aaaacacgataataccatgaagaagcccgaactcac-3'; reverse, 5'-ttttatttttatcgtgtcgacgttaaactcgacctacctccttagcg-3', the *hygro*-encoding sequence and the sequence complementary to the *hygro*-encoding DNA underlined and double-underlined, respectively).

Preparation of rabbit polyclonal antibodies

anti-SARAF pAb

A cDNA fragment encoding SARAF CytD (SARAF195-339 a.a.) was inserted into the EcoRI/SalI site of the bacterial expression vector pGEX-4T-1 (GE Healthcare) by the In-Fusion PCR method using a pair of primers (forward, 5'-tggatccccggaattcagtgacgggcagtattctcc-3'; reverse, 5'-ggccgctcgagtcgactttatcgtctcctggtacca-3'). Using the resultant plasmid pGST-SARAF-Ct as a template, an internal sequence (corresponding to 225-278 a.a.) was deleted by the QuikChange site directed mutagenesis method using a pair of primers (forward, 5'-accaactcagcaggacctcctagcaatagagcggcaacaccc-3'; reverse, 5'-gggtgttgccgctctattgctaggaggtcctgctgagttggt-3') to improve solubility of the expressed recombinant protein GST-SARAF-Ct-4 (expressing 195-224/278-339 of SARAF) in *Escherichia coli*. To further increase the solubility, a DNA fragment encoding GST-SARAF-Ct-4 was transferred to the EcoRI/SalI site of the cold shock vector pColdII (TakaraBio) by the In-Fusion PCR method using a pair of primers (forward, 5'-cgagggatccgaattcatgtcccctatactagg-3'; reverse, 5'-tagactgcaggtcgactttatcgtctcctg-3') and pGST-SARAF-Ct-4 as a template. For expression of maltose-binding protein (MBP)-fused SARAF-Ct-4, pGST-SARAF-Ct-4 was digested with EcoRI and SalI, and a DNA fragment encoding SARAF-Ct-4 was subcloned into the EcoRI/SalI site of pMal-c2

(NEB, Ipswich, MA) to construct pMAL-c2/SARAF-Ct-4.

Escherichia coli BL21 cells were transformed with pColdII/GST-SARAF-Ct-4 and pre-cultured at 37 ºC overnight in LB broth containing 50 μg/mL of ampicillin. Fresh culture was diluted 1:100 with 300 ml of ZYG broth containing 0.4% glucose and ampicillin, and incubated with aeration at 37 ºC. When absorbance of the culture at 600 nm reached 0.3-0.5, culturing temperature was shifted down to 15 ºC. After 30 min, IPTG was added to 0.5 mM, and incubation was continued overnight. Harvested cells were lysed by sonication in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM PMSF, 5 mM benzamidine) containing 1% Triton-X100 and processed for purification with glutathione Sepharose beads (GE Healthcare) essentially according to the provided manufacturer's instructions. After the glutathione-eluted fractions were dialyzed against phosphate buffered saline, the purified GST-SARAF-Ct-4 protein was used for immunization of rabbits.

Escherichia coli BL21 cells transformed with pMAL-c2/SARAF-Ct-4 were cultured at 30 ºC and induced for expression with 0.3 mM IPTG for 3 h. Harvested cells were lysed in lysis buffer (20 mM Tris-HCl, pH7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF) containing lysozyme for 20 min on ice followed by sonication. The MBP-SARAF-Ct-4 protein was affinity purified with an amylose resin column. Maltose-eluted fractions were dialyzed against water and used for immobilization to a HiTrap NHS-activated HP Column (1-mL size, GE Healthcare) according to the manufacturer's instructions. The column was pre-washed with 10 mL of 0.1 M Glycine-HCl, pH 3.0, and renatured with 10 mL of TBS before use. The rabbit antiserum against GST-SARAF-Ct-4 was diluted with TBS and absorbed to the column. After the fractions eluted with 0.1 M Glycine-HCl, pH 3.0, was neutralized with 1 M Tris-HCl, pH 9.0, and dialyzed against PBS, antibodies cross-reacting with GST and MBP were removed by successive incubation with GST-immobilized Sepharose beads and MBP-immobilized amylose resin.

anti-GFP pAb

A cDNA fragment encoding monomeric EGFP (mEGFP; A206K mutant of EGFP, initiation Met not counted for residue Nos.; see Suppl. Refs 4 and 5.) was obtained by PCR-amplification with a pair of primers (forward, 5'-ggttccgcgtggatccatggtgagcaagggcga-3', EGFP-encoding sequence including ATG underlined; reverse, 5'-ggccgctcgagtcgactagtacagctcgtccatgc-3', the nucleotide sequence complementary to the C-terminal EGFP-encoding sequence followed by TAG double-underlined) and using pmEGFP-C1 as a template, and it was subcloned into the

BamHI/SalI site of pGEX-4T-3. The resultant plasmid pGEX-4T-3/mEGFP was used for bacterial expression of GST-GFP in *Escherichia coli* BL21. The BamHI/SalI fragment of pGEX-4T-3/mEGFP was further subcloned into the BamHI/SalI site of pMAL-c2 to construct pMAL-c2/mEGFP, which was used for bacterial expression of MBP-GFP. Bacterial expression and purification of the GST-GFP and MBP-GFP proteins were performed by conventional methods as described elsewhere. While GST-GFP was used for immunization of rabbits, MBP-GFP was immobilized to a HiTrap NHS-activated HP Column (1-mL size) and used for affinity purification of anti-GFP pAb as similarly described for preparation of anti-SARAF pAb.

Supplementary References

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