A STIM2 splice variant negatively regulates store-operated calcium entry

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Cellular homeostasis relies upon precise regulation of Ca$^{2+}$ concentration. Stromal interaction molecule (STIM) proteins regulate store-operated calcium entry (SOCE) by sensing Ca$^{2+}$ concentration in the ER and forming oligomers to trigger Ca$^{2+}$ entry through plasma membrane-localized Orai1 channels. Here we characterize a STIM2 splice variant, STIM2.1, which retains an additional exon within the region encoding the channel-activating domain. Expression of STIM2.1 is ubiquitous but its abundance relative to the more common STIM2.2 variant is dependent upon cell type and highest in naive T cells. STIM2.1 knockdown increases SOCE in naive CD4$^+$ T cells, whereas knockdown of STIM2.2 decreases SOCE. Conversely, overexpression of STIM2.1, but not STIM2.2, decreases SOCE, indicating its inhibitory role. STIM2.1 interaction with Orai1 is impaired and prevents Orai1 activation, but STIM2.1 shows increased affinity towards calmodulin. Our results imply STIM2.1 as an additional player tuning Orai1 activation in vivo.

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Amplicity, duration and kinetics of Ca\(^{2+}\) signals code for differential changes in gene expression, degranulation, cytokine release, proliferation and migration. These diverse functions require tight control and regulation of Ca\(^{2+}\) influx. In immune cells, the major influx pathway for Ca\(^{2+}\) is encoded by components of the store-operated calcium entry (SOCE) machinery. Here, CRAC (Ca\(^{2+}\) release-activated Ca\(^{2+}\)) channels encoded by the Orai genes (Orai1-3) are activated by endoplasmatic reticulum (ER)-localized stromal interaction molecule (STIM) (STIM1 and STIM2) proteins when the Ca\(^{2+}\) concentration of the ER (~400–600 μM) is decreased\(^2\). In contrast to STIM1, the major activator of Orai upon effector T-cell activation, the functional role of STIM2 is less well understood. The first studies on STIM2 in 2006 described an inhibitory effect of STIM2 on STIM1-mediated SOCE, as well as its constitutive and store-independent activation of Orai1 (ref. 3). An short interfering RNA (siRNA)-based screen for regulators of SOCE machinery. Here, CRAC (Ca\(^{2+}\) encoded by components of the store-operated calcium entry (SOCE) model of regulation of basal Ca\(^{2+}\) influx is controversial, and a recent published computational model of regulation of basal Ca\(^{2+}\) oscillations due to partial store depletion, driving Ca\(^{2+}\) oscillations due to partial store depletion, driving Ca\(^{2+}\) and downregulation of CaM or plasma membrane Ca\(^{2+}\)-ATPase increasing basal Ca\(^{2+}\) (ref 4). The authors also showed that STIM2 responds to smaller decreases in ER Ca\(^{2+}\) with an EC50 of 406 μM compared with 210 μM for STIM1 (see also ref. 5). In addition to the decreased affinity of the Ca\(^{2+}\)-binding EF hand, the STIM2 SAM domain displays an increased conformational stability when compared with STIM1 (ref. 5). In contrast to STIM1, the SAM domain of STIM2 contains only a single N-glycosylation site. Modifications of the glycosylation sites within the SAM domain of STIM1 lead to altered oligomerization rates and current size as well described by a reaction diffusion model\(^6\).

Whether the effect of STIM2 on SOCE is activation or inhibition is controversial, and a recent published computational model of regulation of basal Ca\(^{2+}\) homeostasis did not consider a role of STIM2 (ref. 7). A second confounding factor in the analysis of STIM2 function is an existing uncertainty regarding its transcription start site. In the original report describing cloning of STIM2, Williams et al.\(^8\) postulated translation from a non-AUG codon around L88, a view that was later revised by Graham et al., who showed that STIM2 contains an unconventionally long signal peptide with translation from a conserved Met, which can cause incomplete protein translocation into the ER and leads to a small amount of cytosolic STIM2, responsible for pre-coupled and non-store-operated activation of STIM2–Orai1 complexes\(^9\). In addition to its role in maintaining basal Ca\(^{2+}\) and controlling ER Ca\(^{2+}\) levels\(^4\), STIM2 activates Orai1 signalling upon submaximal store depletion, driving Ca\(^{2+}\) oscillations due to partially pre-coupled STIM2–Orai1 population\(^10–14\). STIM2 and Orai1 form a Ca\(^{2+}\)-sensitive and thapsigargin-insensitive complex in cortical neurons\(^15\), with loss of STIM2-protecting neurons from store-mediated hyposcapic neuronal death\(^16\). In cancer, controversial roles for STIM2 as both a tumour suppressor or as a potential oncoene have been postulated\(^17–20\), reviewed in ref. 14). Recent publications also present growing evidence for a key role for STIM2 in tumour immunity by CD8\(^+\) cells\(^21\) and in several immune diseases. In a murine multiple sclerosis model, STIM2-deficient mice were shown to be less susceptible to autoimmune encephalomyelitis (EAE), suggesting a role for STIM2-mediated Ca\(^{2+}\) signalling in autoimmune disease\(^22,23\). In contrast to the EAE model, deficient STIM2 signalling caused salivary gland autoimmune pathology in Primary Sjögren’s Syndrome\(^24\).

Here we report the identification and detailed characterization of a novel STIM2 splice variant, named STIM2.1, which acts as an inhibitory regulator of STIM-mediated activation of Orai channels. The additional exon of STIM2.1 is spliced into the channel-activating domain (CAD), immediately upstream of the sequences essential for binding of Orai1 (refs 13,25), disabling STIM2.1 CAD from activating Orai1 and altering the CAD domain affinity for CaM binding.

Results
Identification of a novel STIM2 splice variant. We discovered the existence of two additional STIM2 splice variants by database mining, namely STIM2.1 containing an additional exon 9 and STIM2.2 containing an alternative exon 13 (13*) leading to an upstream end of translation and a transcript shortened by 444 bp (~17 kDa) (Fig. 1a). All reports on STIM2 are conducted with the STIM2.2 variant. Although we were unable to detect messenger RNA (mRNA) expression of STIM2.3 in lymphocytes by different PCR-based strategies, we identified STIM2.1 in a conventional PCR reaction with primers (for, rev) flanking exon 9. Figure 1b shows two different PCR products in human CD8\(^+\) T cells as well as in Jurkat T cells and primary monocytes. Exon 9-specific quantitative reverse transcription (RT)–PCR primers were derived (Fig. 1a; Supplementary Table 1), PCR products were confirmed by DNA sequencing and relative expression levels of STIM2.1 (with exon 9, NM001169118) and of STIM2.2 (without exon 9, NM020860) were tested using template complementary DNA (cDNA) of naive and stimulated CD4\(^+\) T cells from at least three different primary human blood donors. As also indicated by conventional PCR (inset in Fig. 1c), STIM2.1 expression is highest in naive T cells but is reduced upon stimulation with anti-CD3/anti-CD28-coated beads. Seven hours after bead contact, the ratio of STIM2.2/STIM2.1 transiently increases to 4 ± 0.5, but decreases again to a ratio of 1.6 ± 0.56 after 72 h following initial bead contact (Fig. 1c). A reduction of mRNA expression is also seen for STIM2.2 and for STIM1 although STIM1 expression recovers after 72 h, whereas total STIM2 mRNA and STIM2 protein remains reduced in stimulated cells (Fig. 1c; Supplementary Fig. 1a,b). We proceeded to test splice-specific expression in a number of cell lines and tissues and plotted the ratio of STIM2.2/STIM2.1 expression over STIM2.1 expression (Fig. 1d). Highest expression of the novel STIM2.1 (lowest ratio) is detected in naive CD4\(^+\) and CD8\(^+\) T cells. cDNA from glioblastoma samples (12 patients) showed the highest expression of STIM2.2 with little STIM2.1. In summary, we could detect expression of STIM2.1 in all tested human cell lines and primary cells, although expression was always lower than STIM2.2. Alignment of the exon 9 amino acids (VASSYLIQ) shows a high degree of conservation between different species. We also tested for STIM2.1 (VAASYL)IQ expression in lymphocytes from Mus musculus and were able to detect two bands by conventional PCR (Supplementary Fig. 1d).

Knockdown of STIM2.1 alters SOCE in primary cells. Because naive CD4\(^+\) and CD8\(^+\) T cells showed the highest absolute expression of STIM2.2/STIM2.1 expression of 1.6 ± 0.4 (5 donors) for naive CD4\(^+\) cells and a ratio of 1.5 ± 0.14 (3 donors) for CD8\(^+\) cells (see Fig. 1c), these cells lent themselves for investigating endogenous STIM2.1 function. Although limited by the very short sequence of exon 9, we devised splice-specific siRNA targeting either exon 9 or the exon 8/exon 10 boundary (Supplementary Table 1). Efficiency of knockdown was tested by qRT–PCR 14–18 h after siRNA transfections on two consecutive days. STIM2.1-specific siRNA decreased cDNA expression to an average of 44 ± 6% of its non-silenced control without exerting a significant effect on the expression of STIM2.2 or on STIM1 (Fig. 2a), neither did the siRNA show off-target or indirect effects on the expression of Orai1 (97% of control). Given an mRNA
expression ratio of STIM2.2/STIM2.1 of 1.6 and a knockdown efficiency of ~50%, we expected a reduction of total STIM2 protein of about 20%, which we indeed observed (Supplementary Fig. 1c). Splice-specific knockdown of STIM2.2 was not as successful, leading to reduction of STIM2.2 expression to an average of 68 ± 8% and to small (~10%) off-target or indirect effects on STIM1, STIM2.1 (Fig. 2b) and Orai1 (91% of control) expression. Measurements of [Ca^{2+}]_i of naïve T cells showed that siRNA specific to STIM2.1 did not have a significant effect on basal [Ca^{2+}]_i; however, it led to a significant increase in rate, peak and plateau of SOCE (Fig. 2c–g). In contrast and despite the relatively weak downregulation of STIM2.2 expression, Ca^{2+} imaging of primary naïve CD4^{+} cells revealed that STIM2.2 siRNA significantly decreased basal Ca^{2+} concentrations in bath solutions with different [Ca^{2+}]_o (Fig. 2h,i), as expected upon downregulation of STIM2 (ref. 4), decreased the size of the thapsigargin (Tg)-releasable pool, which is very small and variable in naïve T cells (see Supplementary Fig. 1e) and significantly reduced SOCE (Fig. 2j–l), demonstrating that expression of STIM2.2 in naïve cells is a significant determinant of their Ca^{2+} homeostasis, and confirming that STIM2.2 is a regulator not only of basal but also of store-operated Ca^{2+} homeostasis. STIM2.1 siRNA treatment showed opposite effects, strongly indicating that STIM2.1 acts as a break or inhibitory regulator on STIM1/STIM2-mediated SOCE in vivo.

Upregulation of STIM2.1 decreases T-cell SOCE. If downregulation of STIM2.1 increases SOCE, we reasoned that upregulation of STIM2.1 in Jurkat T cells may decrease SOCE and therefore expressed either STIM2.2 or STIM2.1 after cloning exon 9 into a STIM2 expression vector with the full STIM2 signal peptide sequence and where the fluorescent mcherry-coding sequence replaced the variable domain NT 1966–2160 (I648-K711), retaining 121 C-terminal aa residues (see also below). Figure 3a shows that overexpression of STIM2.2 in Jurkat T cells leads to an increased basal [Ca^{2+}]_i, a reduced Tg-releasable store content but also an increased rate, peak and plateau of SOCE (Fig. 3b–e). Overexpression of STIM2.1 showed similar effects to STIM2.2 on basal Ca^{2+} and Tg-releasable store content but, in contrast to STIM2.2, significantly decreased rate, peak and plateau of SOCE (Fig. 3c,e). These results complement the siRNA data concerning SOCE (Fig. 2) and confirm an opposite role for both splice variants in regulation of SOCE after full store depletion.

STIM2.1 is unable to activate Orai1. To further analyse the function of STIM2.1, we cloned exon 9 into an existing STIM2.2 expression vector, where the STIM1 signal peptide sequence is followed by YFP and the sequence of STIM2 starting at C102

Figure 1 | Identification of a novel STIM2 splice variant. (a) Schematic representation of human STIM2 mRNA with exon boundaries. Highlighted in red are exon 9 and 13^{*} present only in the splice variants STIM2.1 and STIM2.2, respectively. The enlarged region shows the primer pairs used in conventional (for: grey arrow and rev: blue arrow) or quantitative (qPCR for, qPCR rev – 9, qPCR rev + 9) PCRs to detect and analyse splice variant expression. (b) Image showing PCR amplification products obtained with primer pair (for and rev) using cDNA from naïve and stimulated human CD8^{+} T cells, Jurkat T cells and human monocytes. (c) Relative expression of STIM1, STIM2.1 and STIM2.2 in naïve and stimulated CD4^{+} T cells with indicated time periods after initial contact with anti-CD3/anti-CD28-coated beads. Expression was normalized to that of TBP (three donors) (for: grey arrow and rev: blue arrow) or quantitative (qPCR for, qPCR rev – 9, qPCR rev + 9) PCRs to detect and analyse splice variant expression. (d) Ratio of STIM2.2/STIM2.1 expression obtained by qRT-PCR using reverse-transcribed mRNA isolated from the cell types indicated below the bars (3–12 donors or independent RNA preparations).
Figure 2 | Specific knockdown of STIM2.1 alters SOCE in primary cells. (a) Relative expression of STIM1, STIM2.1 and STIM2.2 after transfection with siRNA against exon 9 (STIM2.1) red or siRNA against exon 8/10 boundary (b, STIM2.2, blue), corresponding control transfection, black bars. (c) Traces showing average changes in intracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)\(_i\)], over time in CD4\(^+\) T cells transfected with control (black, n = 698) or siRNA against STIM2.1 (red, n = 579) in response to perfusion of different [Ca\(^{2+}\)\(_o\)]. (d-g) Quantification of changes in [Ca\(^{2+}\)\(_i\)], measured in c. (h) Traces showing changes in [Ca\(^{2+}\)\(_i\)], in CD4\(^+\) T cells treated as in c after transfection with control (black, n = 978) or siRNA against STIM2.2 (blue, n = 776). (i-l) Quantification of changes in [Ca\(^{2+}\)\(_i\)], measured in h. Measurements shown in e-l are obtained from cells of three independent blood donors and ≥8 experiments. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s T-test. Data obtained are presented as mean ± s.e.m.

Figure 3 | Upregulation of STIM2.1 decreases T-cell SOCE. (a) Traces showing average changes in intracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)\(_i\)], over time in Jurkat T cells transfected with control (black), STIM2.1-mCherry (red) or STIM2.2-mCherry (blue) in response to perfusion of different [Ca\(^{2+}\)\(_o\)], indicated in the upper bar. (b-e) Quantification of changes in [Ca\(^{2+}\)\(_i\)], measured in a from 131 to 216 cells of 6 experiments. **P < 0.01, ***P < 0.001, Student’s T-test. Data obtained are presented as mean ± s.e.m.

(ref. 4) or into a vector containing the full STIM2 signal peptide (M1-G101) and coding sequence with no added fluorescent tag\(^{10}\). Expression of each of these constructs in HEK293 cells stably expressing Orai1 (HEK01) cells revealed a significant increase in the basal Ca\(^{2+}\) content of the cytosol upon expression of STIM2.2, as well as a significant increase in SOCE when
compared with an equimolar vector control. Figure 4a–e shows the results for the N-terminally tagged constructs (see also ref. 4). Expression of STIM2.1 alone did not increase basal $[\text{Ca}^{2+}]_i$, nor significantly affected the Tg-releasable pool. However, in contrast to STIM2.2, STIM2.1 is not only unable to activate SOCE upon store depletion but also acts as an inhibitor of SOCE in the vector-only-transfected control (Fig. 4a–e). The YFP or mcherry fluorescence of the directly tagged constructs (Fig. 3) clearly indicated expression, which was also confirmed by western blot analysis (Supplementary Fig. 1f). To directly measure channel activity, we conducted whole-cell patch-clamp recordings of STIM2.1 and STIM2.2 expressed in HEKO1 cells with a pipette solution containing IP$_3$ and BAPTA (1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid). As expected, STIM2.2 displayed inward currents that showed an initial rapid phase (store operated) and a second slower phase of increasing current density indicative of loss of a diffusive factor from the cytosol$^{10}$ (Fig. 4f). Similar to the results shown in Fig. 4a, STIM2.1 was unable to sustain Orai1 currents (Fig. 4g–h). We also tested the ability of STIM2.1 and STIM2.2 to activate Orai2 channels and obtained a related result, namely a large increase in basal Ca$^{2+}$ and Orai2-mediated SOCE can be observed with STIM2.2 but not with STIM2.1 (Supplementary Fig. 1g–k). The results shown in Fig. 4 indicate that STIM2.1 by itself either may be unable to cluster and localize to ER–PM (plasma membrane) junctional regions, unable to bind to and/or unable to gate Orai1 channels effectively. Thus, we set out to investigate the mechanisms underlying the observed functional differences between STIM2.1 and STIM2.2.

**STIM2.1 interaction with Orai1 is impaired.** We used total internal reflection fluorescence (TIRF) microscopy to assess the ability of STIM2.1 to oligomerize, translocate towards the ER–PM junctional regions, to recruit Orai1 to STIM2 punctae and to form clusters with functional coupling between Orai1 and STIM2.2. These results explain the inability of STIM2.1 to activate Orai1, but also indicate that either a second interaction site is unperturbed (leading to co-localization) or that an interaction still exists but that the distance between the fluorescent tags now is too far to measure FRET. Controls and correction factors are described in detail in the Methods section. As shown in Fig. 5d, both STIM2.2 and STIM2.1 formed extensive punctae also without stimulation, as predicted from the lower EF-hand Ca$^{2+}$ affinity of STIM2, pointing towards normal luminal Ca$^{2+}$ sensing and oligomerization mechanisms within STIM2.1. The fact that most of these clusters do not contain Orai1 indicates that either STIM2 is not yet completely unfolded or possibly that additional cytosolic factors prevent extensive Orai1 co-clustering. Nevertheless, the high resting calcium (Fig. 4) with overexpression of STIM2.2 indicates the presence of clusters with functional coupling between Orai1 and STIM2.2.

**STIM2.1 opposes STIM2 and STIM1 function.** Given the results shown in Figs 2 and 3, we also investigated heterologous co-

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**Figure 4 | STIM2.1 is unable to activate Orai1 in overexpression systems.** (a) Traces showing average changes in intracellular Ca$^{2+}$ concentration $[\text{Ca}^{2+}]_i$, over time in response to perfusion of different $[\text{Ca}^{2+}]_i$, indicated in the upper bar in HEKO1 cells transfected with YFP-STIM2.1 (red, $n = 96$) or YFP-STIM2.2 (blue, $n = 107$) or an empty vector control (grey, $n = 72$), 6 experiments per condition. (b–e) Quantification of changes in $[\text{Ca}^{2+}]_i$, measured in a. (f) Average traces showing whole-cell current density (CD) over time extracted at $-130$ mV in HEKO1 cells transfected with YFP-STIM2.1 (red) or YFP-STIM2.2 (blue). (g) Average maximum CD recorded from cells measured in f. (h) Current-voltage ($I$–$V$) relationship of representative cells recorded in f. ***$P < 0.001$, Student’s $T$-test. Data obtained are presented as mean ± s.e.m.
expression and co-transfected HEKO1 cells with STIM2.1 together with STIM2.2, STIM1 or an equimolar amount of YFP containing a control vector. Co-overexpression of STIM2.2 and the control vector in HEKO1 cells results in an extremely high basal Ca\textsuperscript{2+}, indicating indeed a high activity of coupled STIM2.2 and Orai1. Nonetheless, STIM2.2 still elicits additional activation of Orai1 after store depletion in 0 mM Ca\textsuperscript{2+} (Fig. 6a–e). Keeping STIM2.2 constant and co-expressing STIM2.1 significantly decreased pre-coupled and full-depletion-operated Ca\textsuperscript{2+} (Fig. 6a–e). Analysed cells were not sorted for preactivated cells. We also performed whole-cell patch-clamp recordings of HEKO1 cells co-transfected with either splice variant alone and equimolar vector control or with STIM2.2 and STIM2.1. In contrast to the imaging results, co-expression of STIM2.1 did not dampen STIM2.2-mediated activation of Orai1 in conditions of extreme intracellular Ca\textsuperscript{2+} buffering (20 mM BAPTA; IP\textsubscript{3}) (Fig. 6f,h,i). To more closely mimic the Ca\textsuperscript{2+}-imaging condition with only very slightly buffered intracellular Ca\textsuperscript{2+} (Fura2), we clamped the intracellular free Ca\textsuperscript{2+} concentration to 150 nM with addition of IP\textsubscript{3} and repeated the patch-clamp analysis (Fig. 6g,j,k). Now co-expression showed a significant inhibitory effect in the initial store-operated phase (grey box in Fig. 6g): between 12 and 90 s after break-in, 32 of 41 time points showed significantly \((P<0.05)\) reduced current densities \((n=22\) cells for each condition) with co-expression of STIM2.1 and STIM2.2. Expression of STIM2.1 did not elicit currents in either condition. Analyses of maximal current densities and exemplary IV relations are shown in Fig. 6h–j. Primary human CD4\textsuperscript{+} T cells as well as Jurkat T cells used in Figs 2 and 3 express both STIM1 and STIM2 (Fig. 1); therefore, we also analysed co-overexpression of STIM2 splice variants with STIM1. Co-expression of STIM2.2 with STIM1 in HEKO1 cells leads to an expected significant increase in basal \([\text{Ca}^{2+}]_i\) (Supplementary Fig. 3a); however, STIM1-mediated store depletion-induced Ca\textsuperscript{2+} entry (green trace) was not much affected by co-expression of STIM2.2 analysed within 24 h after transfection (Supplementary Fig. 3a–e). In contrast, while co-expression of STIM2.1 together with STIM1 did not have a significant effect on basal \([\text{Ca}^{2+}]_i\), it significantly inhibited SOCE (Supplementary Fig. 3a–e) thus confirming a negative regulatory role of STIM2.1 in conditions of full store depletion as also seen for naive CD4\textsuperscript{+} T cells and Jurkat T cells (Figs 2c,g and 3). Whole-cell patch-clamp recordings of HEKs1 cells (stable STIM1 expression), with co-overexpression of Orai1 together with vector only, STIM2.2 or STIM2.1 revealed that in strong buffering conditions (20 mM BAPTA, IP\textsubscript{3}) co-expression of both STIM2.2 and STIM2.1 increased STIM1-mediated Orai1 current densities (Supplementary Fig. 3f–h). Clamping \([\text{Ca}^{2+}]_i\), to 150 nM in HEKO1 cells upon co-expression of STIM1 with STIM2.1 or STIM2.2 showed smaller currents with very variable kinetics, thus potential inhibitory effects may be difficult to detect (Supplementary Fig. 3i–k). However, both sets of patch-clamp results (Fig. 5; Supplementary Fig. 3) confirm a direct or indirect Ca\textsuperscript{2+}-dependent effect in STIM2.1’s ability to act as an inhibitory subunit of SOCE.

**Homology modelling and protein–protein docking analysis.** To correlate the observed phenotype of STIM2.1 to available structural information on STIM1, we applied homology modelling to predict the structures of STIM2.2 and of STIM2.1 carrying the VAASYLIQ insertion. Homodimers of STIM2.2 and STIM2.1 were modelled according to the structure of the STIM1 dimer (PDB ID: 3TEQ). Details on the applied algorithms and methods as well as some additional validation are given in the Supplementary Material. One certainly needs to acknowledge that, in the case of STIM2.1, the accuracy of modelling such a
Figure 6 | STIM2.1 opposes STIM2.2 function. (a) Traces showing average changes in intracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_{i}\), over time in response to perfusion of different [Ca\(^{2+}\)]\(_o\), indicated in the upper bar in HEK01 cells co-transfected with 0.5 μg CFP-STIM2.2 together with YFP-control vector (blue) or 1 μg YFP-STIM2.1 (purple). (b–e) Quantification of changes in [Ca\(^{2+}\)]\(_i\), measured in a from 66–69 cells of 6 experiments. (f) Average traces showing whole-cell current density (CD) over time extracted at –130 mV in HEK01 cells co-transfected like in a or with 1 μg YFP-STIM2.1 (red, same trace as Fig. 4f) with internal solution clamping [Ca\(^{2+}\)]\(_o\) to 0. (g) Average traces showing whole-cell CD over time measured from cells transfected like in f with an internal solution clamping [Ca\(^{2+}\)]\(_o\) to 150. Grey box denotes time points between 12 and 90 s after break-in. (h) Average maximum CD recorded from cells measured in f and g, respectively. (i) Current-voltage (I-V) relationship of representative cells recorded at maximal current in f and g, respectively. **P<0.01, ***P<0.001, Student’s T-test. Data obtained are presented as mean ± s.e.m.

large insertion by homology modelling is somewhat limited. Thus, we have considered four alternative cases for STIM2.1 (Fig. 7a; Supplementary Fig. a) where the 8 amino-acid insertion leads either to an ideal extension of helix S2z1 (model 1), a flexible loop (model 2) or a partial helix (models 3 and 4). Upon docking these dimer models of the STIM CAD domains to the Orai1 C-terminal helix using the three different docking packages DOT2 (ref. 27), FRODOCK28 and ZDOCK29, this extension turned out to have a significant, consistent impact on the predicted Orai1-binding sites. For the STIM1 CAD dimer, all best-scoring solutions generated by the packages predicted the Orai1 C-terminal helix to bind to motif 1 of STIM1 (Fig. 7b). In contrast, Orai1 was predicted to favour binding to motifs 2 and 3 of STIM2.2 and of the four STIM2.1 models (Fig. 7b; Supplementary Fig. 4b). Note that due to the symmetric dimer geometry, some configurations docked to the left or right monomer (mostly in motif 3) are in fact (almost) equivalent. On the basis of the proposed model of STIM1–Orai1 coupling reviewed in ref. 13, the C-terminal helix of Orai1 can access the upper part of the CAD domain but hardly is able to approach its lower part, which is close to the dimerization domain (Dd) of CAD. Thus, the postulated docking positions in motifs 2 and 3 are likely not biologically active. To rationalize the docking results, we calculated the electrostatic potential around all CAD domains and the Orai1 C-terminal helix with the software APBS30 and plotted the potential on the corresponding solvent-accessible surfaces. As shown in Supplementary Fig. 5, a large fraction of the surface area of Orai1 helix is negatively charged. To make energetically favourable interactions, the Orai1 helix should align its negatively charged surface with the positively charged strip on the STIM1 CAD domain. This is likely the reason why the Orai helix was preferably oriented in the upper part of the STIM1 CAD domain termed motif 1 by the three programs (Fig. 7b). Compared with STIM1, the positively charged areas around motif 1 shrink in size in the STIM2.2 and STIM2.1 CAD domains (Fig. 7c; Supplementary Fig. 4c). Besides, in models 2, 3 and 4 of STIM2.1 CAD, the protrusion of the loop (or partial loops) in motif 1 made the interaction surfaces less smooth than the surface of an ideal helix. As a result, alternative positions for the Orai1 helix (near motifs 2 and 3) were predicted to be energetically more favourable for binding to the STIM2.2 and STIM2.1 models (Fig. 7b; Supplementary Fig. 4b). As argued above, these alternative positions are likely biologically not plausible. Figure 7c shows that the electrostatics of the Dd of STIM2.1 model 1 CAD is quite different from that of STIM1 and STIM2.2. Precisely, the Dds of STIM1 and STIM2.2 CAD are
relatively neutral, whereas the domains are more positively charged in STIM2.1 model 1. This suggests that STIM2.1 CAD may not function as a stable dimer due to repulsion between its equally charged domains.

Biochemistry of the STIM2.1 and STIM2.2 CAD domains. To obtain further biochemical evidence of splice variant differences and, as the insertion altered the scores of predicted calmodulin (CaM)-binding sites\(^1\), we tested the amino acids relevant for binding of the Orai1 C-terminal sequence to both the STIM2.2 and to the STIM2.1 CAD domains by probing immobilized peptide sequences either with \(^{14}\)C-labelled glutathione transferase (GST-CaM) or with a biotinylated Orai1 C-terminal peptide. Each peptide spot contains a 15-amino-acid sequence starting with Y452 (translated from first ATG (NT 268) of NM001169118.1). The subsequent spot started with a three amino-acid shift generating spots with an overlap of 12 identical amino acids (aa) to the next neighbour (Supplementary Fig. 6). Figure 8 shows images of membranes after phosphoinosferase development (Fig. 8a) or chemiluminescence detection (Fig. 8b). Analysis of resulting signals shows that biotinylated Orai1 binds with high specificity to spot 12 (KRSTVFGTHV AHSS) present in both splice variants (spot 41 is identical, see Supplementary Methods). Both the preceding spot 11 (IKKKRSTVFGTHV) and the following spot 13 (TVFGTHV AHSSSLD) show nearly no binding, indicating that the presence of K477 and R478 as well as of H489 (highlighted in bold in the above sequence of spot 12) is likely critical for Orai1 binding to STIM2. Binding of CaM shows splice-specific differences: Here inclusion of the 8 aa adds an almost classic CaM-binding motif (IQxxKIXxxRxV) present in STIM2.1 spot 9 (LIQAEKIKKRSVV). STIM2.2 sequences lack this motif but also bind well to CaM with highest binding to spot 36 (MQLAIKDEAEIKK) (Fig. 8c,d). Spots 45–50, derived from a putative in silico CaM-binding site located upstream of the transmembrane domain, display only weak CaM binding. Overlay of both Orai1- and CaM-binding motifs on the linear sequence indicates partial overlap of these sites, indicating that binding of either Orai1 or CaM may be mutually exclusive (Fig. 8d). To further analyse CaM binding, we expressed STIM2.1 and STIM2.2 CAD domains in Escherichia coli, purified and partially refolded the proteins and binding to CaM with CaM-sepharose pull-down assays in buffers containing Ca\(^{2+}\) or EGTA. Supplementary Figure 7 shows that both STIM2 CAD domains bind efficiently to CaM-sepharose in a Ca\(^{2+}\)-dependent manner (Supplementary Fig. 7a). With subtraction of background (agarose bound) signal, we uncovered a small difference in binding between the variants (Supplementary Fig. 7b). We performed surface plasmon resonance spectroscopy (SPR) to determine relative affinities for binding of CAD domains to immobilized GST-CaM (Fig. 8e–g). No binding was observed in the absence of Ca\(^{2+}\), confirming results shown in Supplementary Fig. 7. However, in the presence of Ca\(^{2+}\), the STIM2.1 CAD domain shows a significantly higher affinity with a binding constant of 9.1 ± 2.7 nM compared with 40.7 ± 10.5 nM for STIM2.2. These results suggest that STIM2.2 and STIM2.1 are affected by both the local Ca\(^{2+}\) and CaM concentration. However, even with a mutated IQ motif, STIM2.1 is unable to activate Orai1 (unpublished data).

Discussion

In our study, we report the identification and functional analysis of a hitherto unknown STIM2 splice variant, STIM2.1, where the sequence VAASYLQ is spliced into the CAD/SOAR (Sx1) domain. We show that STIM2.1 is expressed in many cell types, where its relative expression ratio to the known variant STIM2.2 can differ up to eightfold in the cell types we tested. siRNA-mediated downregulation of STIM2.1 in primary naive human CD4\(^{+}\) cells, which express a high amount of both STIM2 splice variants (Fig. 1), shows that thapsigargin-induced SOCE is increased with no effect on basal Ca\(^{2+}\). Given the 44% remaining relative expression level of STIM2.1 in the siRNA-transfected cells, complete knockout of STIM2.1 is very likely to display an
Figure 8 | Characterization of Orai1 and Calmodulin-binding properties of STIM2.1 and STIM2.2. (a) 14C-labelled calmodulin (CaM) binding to STIM2 peptide arrays. (b) Biotinylated Orai1 C-term peptide binding to identical STIM2 peptide array as in a. Spots encircled with a red dotted line represent peptides encoding exon 9 sequences, blue dotted line represent peptides encoding the sequence flanking exon 9 (exon 8/10 transition unique in STIM2.2) and black circles represent peptides encoding conserved sequence in both splice variants. (c) Quantification of bound Orai1 peptide (black bars) or CaM (white bars) to the different peptide spots. (d) Schematic representation of partial sequences of STIM2.1 (upper sequence) or STIM2.2 (lower sequence) encoded by the spotted peptides, showing the domains binding to CaM in STIM2.1 (yellow, encoded by peptides 7–9 and 16–17) and in STIM2.2 (yellow, encoded by peptides 35–38) and the identical Orai1-binding domain (red encoded by peptide 12 for STIM2.1 and peptide 41 for STIM2.2). (e,f) Surface plasmon resonance (SPR) analysis of the binding affinity of calmodulin with representative binding curve sensorgrams showing real association and dissociation of purified CaM domains of STIM2.2 (red-filled boxes denote differences to STIM1). (e) or STIM2.1 (f) with immobilized CaM. (g) Average Ks values for CaM binding of STIM2.2 (blue) or STIM2.1 (red) obtained from five independent experiments using CAD concentrations ranging from 7.5 to 750 nM, *P < 0.05, Student’s T-test. Data obtained are presented as mean ± s.e.m.

even stronger amplification of SOCE and may uncover a phenotype on basal \( \text{Ca}^{2+} \). Basal \( \text{Ca}^{2+} \) levels increase during T-cell activation\(^2\) and although it is tempting to speculate that this increase in basal \( \text{Ca}^{2+} \) is due to decreased levels of STIM2.1, expression of both STIM2.2 and STIM2.1 decreases after bead contact and remains low in effector cells, whereas STIM1 expression recovers after 48–72 h and now is stronger than the sum of both STIM2 splice variants (Fig. 1c). In naïve cells, downregulation of STIM2.1 is in contrast to the splice-specific downregulation of STIM2.2, which reduced both basal \( \text{Ca}^{2+} \) as well as SOCE (Fig. 2). Database mining\(^3\) indicates that CaM is also more highly expressed in naïve than in effector CD4\(^+\) cells. Interestingly, overexpression of both variants in Jurkat T cells (Fig. 3) leads to a slight increase in basal \( \text{Ca}^{2+} \). The effects upon downregulation (Fig. 2) and overexpression (Figs 3 and 6; Supplementary Fig. 2) suggest that STIM2.1 acts as a negative regulator of both STIM1 and STIM2.2 only in full store depletion-mediated activation of Orai1. The high expression of STIM2.1 (Fig. 1) in primary CD4\(^+\) T cells thus may reduce sensitivity towards stimulatory factors and likely aids in maintaining naive cells in an inert state. The inhibitory effect of co-expression in \( \text{Ca}^{2+} \)-imaging experiments is not apparent in whole-cell patch-clamp recordings when cells are dialysed with high amounts of BAPTA, although single STIM2.1 expression is still unable to activate Orai1 in such conditions (Fig. 4), consistent with its lack of interaction seen in FRET experiments (Fig. 5). Homology modelling suggests that STIM2.1 shows altered binding configurations and may not form stable CAD dimers due to electrostatic repulsion of its Dds (Fig. 7). Clamping cytosolic \( \text{Ca}^{2+} \) to 150 nM in presence of IP\(_3\) unmasked a dominant-negative effect of STIM2.1 on the store-operated phase of STIM2.2-mediated currents and abolished amplification of STIM1 currents (Supplementary Fig. 2i–k). Together, these results suggest that either heterodimers of STIM2.1 with STIM2.2 and possibly with STIM1 can become functional in conditions of extreme buffering (high BAPTA, \( 0 \text{[Ca}^{2+}] \)) or that STIM2.1 precludes binding of STIM2.2 or STIM1 to Orai1 subunits by steric hindrance only if cytosolic \( \text{Ca}^{2+} \) ions are present, the latter we believe to be more likely. A differential \( \text{Ca}^{2+} \) requirement for steric hindrance may explain why STIM2.1’s inhibitory effect on basal \( \text{Ca}^{2+} \) is not always seen upon overexpression (compare basal \( \text{Ca}^{2+} \) of Figs 3 and 4 with...
Fig. 6) and would also indicate a clear dependency on its expression level. Direct binding of Ca$^{2+}$ to the inhibitory domain of STIM1 has been reported$^{33,34}$, and local cytosolic Ca$^{2+}$ elevations are required for STIM1 deoligomerization and termination of store-operated Ca$^{2+}$ entry$^{35,36}$; but it is unclear how cytosolic Ca$^{2+}$ affects STIM2 oligo- or deoligomerization. Parvez et al.$^{10}$ showed that STIM2 shows a biphasic activation of Orai1 with an early store-dependent phase and a slower and store-independent activation due to loss of CaM through intracellular dialysis (see also Fig. 4). Our comparison of STIM2.1 and STIM2.2 CAD domain binding to CaM using SPR shows that both domains bind to Ca$^{2+}$-CaM with a much higher affinity than the C-terminal site (10 and 40 nM versus $\sim 1\mu M$) of STIM2.1. STIM2.1 has an increased affinity and an altered CaM-binding motif likely generated in part by the specific aa sequence (IQ). We also show that Orai1- and CaM-binding sites overlap on a linear peptide array, giving biochemical evidence to the hypotheses of Parvez et al.$^{10}$ that CaM binding to STIM2 can prevent its activation of Orai1. However, the fact that STIM2.1 by itself is unable to activate Orai1 even after long dilution of cytosolic factors in the presence of BAPTA, or upon mutation of its IQ motif (unpublished data) argues against CAM binding and permanently occluding the Orai1-binding site. STIM2.1 can still clearly co-localize with Orai1 upon full store depletion (Fig. 5), but the tight interaction with Orai1 required for gating is impaired (FRET). The reduced interaction may be caused by the electrostatic destabilization of STIM2.1 dimer formation and of the Orai1-binding site predicted by molecular modelling analyses (Fig. 7).

Because basal [Ca$^{2+}$], is significantly increased with expression of STIM2.2 in HEK01 cells independent of the nature of its signal peptide (short STIM1 or long STIM2), it is highly unlikely that this constitutive entry is mediated by non-ER-localized STIM2 as postulated by Graham et al.$^9$. Besides the steric and electrostatic implications derived from our molecular modelling approach (Fig. 7), the insertion of VAASYLQ can also be compared with the effects of mutating amino acids in a similar upstream position relative to the Orai1-binding site in the STIM1 CAD domain: a STIM1 A376 (corresponding to STIM2.2 A467 = Y in STIM2.1, see box 1 in Fig. 8d) to K mutant constitutively forms punctate at resting ER Ca$^{2+}$ with no ability to recruit Orai1 to these ER–PM sites$^{38}$. Moreover, a STIM1 L373 (= L464 in STIM2.2, = A in STIM2.1 see box 2 in Fig. 8d) to S mutant fails to couple and activate Orai1 currents$^{39}$. Insertion of VAASYLQ thus displays a phenotype that is comparable to certain STIM1 mutants within the S21 helix of SOAR/CAD. In addition, a very recent report$^{40}$ describes the interaction between CC1 domain preceding the CAD domain and CAD CC3 domain of STIM1 as a critical regulatory mechanism affecting exposure of the CAD CC2 domain, which may also be affected in the case of STIM2.1.

The identification of a STIM2 splice variant antagonizing STIM1 function resets the stage for analysis of contradictory results regarding the physiological role of STIM2 (that is, in cancer and autoimmunity$^{14}$) as all studies involving downregulation or knockout would affect both variants, thus dampening a ‘true' STIM2.2-mediated effect.

**Methods**

**Cell culture and antibodies.** All cells were maintained in a 37°C, 5% CO$_2$ humified incubator in corresponding medium, namely, minimum essential medium for HEK293 WT (HEK) and stably expressing Orai (HEKO1), AMIV for human CD4$^+$ cells and RPMI 1640 for E6.1 Jurkat T (Jurkat) cells. All media were supplemented with 10% fetal calf serum and 1% penicillin–streptomycin and HEKO1 were maintained in 1 μg/ml puromycin. HKEE cells were passaged by treatment with trypsin/EDTA. For transfection, the indicated amount of DNA was electroporated into HEK cells and Jurkat cells with Nucleofector II or into CD4$^+$ cells with 4D Nucleofector core unit according to the manufacturer’s instructions.

16–20 h before measurements. Human Orai1, Orai2 and STIM1 were subcloned into the ECORV site of either RFP pMAX, or IRES-RFP-pCAGGS. STIM2 was purchased with an N-terminal GFP or YFP tag (Addgene). We used this cDNA (STIM2.2: PEX-CMV-SP-YFP-STIM2) and two complementary primers (see Supplementary Table 1) each encoding the exon 9 sequence to insert the desired nucleotides into the exon 8/10 boundary. All constructs were confirmed by sequencing. Antibodies used in this study were anti-His Tag (Cell Signaling, 2366, 1:1,000) and anti-STIM2 (C-term, off-target, anti-STIM2 (C-term, off-target) and anti-y-tubulin (Cell Signaling, #S886, 1:1,000).

**Quantitative real-time PCR.** For qRT–PCR, the indicated cell types were harvested in TRIZol (Life Technologies) and stored at −80°C until RNA was isolated following the manufacturer’s instructions. SuperScriptTM III Reverse Transcriptase (Life technologies) was used to generate cDNA and subsequent PCR or qRT–PCR was conducted using the QuantiTect SYBR Green Kit (Quagen) and a CFX96 Real-Time PCR System (Bio-Rad) with the primers listed in Supplementary Table 1. For quantification, expression levels are presented as the normalized quantization cycle (Cq) values of the gene of interest to that of TBP (TATA box-binding protein) using the ACT method (results were comparable when normalized to RNA polymerase).

**Small interfering RNA knockdown.** Splice-specific siRNA targeting exon 9 or exon 8/10 boundary was used to specifically knock down endogenous STIM2.1 or STIM2.2, respectively. Using electroporation as mentioned above, one μM stock solution of the indicated siRNA or an equivalent concentration of non-targeting RNA was transfected in parallel for the non-silencing control were transfected into naive CD4$^+$ cells. Transfection was repeated on the next day and measurements were conducted 14–18 h later. The sequences of the siRNA are listed below. Knockdown efficiency and off-target effects were tested by qRT–PCR, where the mRNA level was normalized to TBP. Sequences of the used siRNA and qRT–PCR primers are listed in Supplementary Table 1.

**Electrophysiology.** Recordings were performed at room temperature in the tight-seal whole-cell configuration, and linear voltage ramps from −150 to +150 mV were applied as in ref. 6. The pipette solution contained the following (in mM): 120 Cs-glutamate, 3 MgCl$_2$, 20 Cs-BAPTA, 10 Hepes and 0.05 IP3 (pH 7.2 with CsOH). Where indicated, internal solution contained 150 μM free Ca$^{2+}$. The external solution contained (in mM): 120 NaCl, 10 TEA-Cl, 2 MgCl$_2$, 10 Hepes and glucose (pH 7.2 with NaOH).

**Fluorescence-based Ca$^{2+}$ imaging.** Human CD4$^+$ or Jurkat T cells were loaded in suspension with 1 μM Fura 2-AM at room temperature for 25 min and seeded on poly-ornithine-coated glass coverslips. HEK01 cells were loaded at 37°C for 25 min with slight rotation on an orbital shaker. All experiments were performed using a self-built perfusion chamber with low volume and high solution exchange rate at room temperature. The external Ca$^{2+}$ Ringer solution contained (in mM): 155 NaCl, 2 MgCl$_2$, 10 glucose, 5 Hepes and 0.5 CaCl$_2$ (0.5 Ca$^{2+}$ Ringer) or no CaCl$_2$, but 1 EGTA and 3 MgCl$_2$ instead (0 Ca$^{2+}$ Ringer) (pH 7.4 with NaOH). Images were analysed with TILLvision software. The absolute intracellular Ca$^{2+}$ concentration was estimated from the relation (Ca$^{2+}$) = $K_d$ (FreeCa$^{2+}$)/(R$_{m}$ - R$_{off}$) where the values of $K_{off}$, R$_{on}$ and R$_{off}$ were determined from an in situ calibration of Fura-2 AM in Jurkat T cells as described in ref. 41. Quantification of the trace shows the average basal [Ca$^{2+}$], the maximal Tg-induced peak in [Ca$^{2+}$], maximal and plateau [Ca$^{2+}$], upon readout of 0.5 mM [Ca$^{2+}$], and influx rate of cells. The minimal [Ca$^{2+}$], before addition of Tg or 0.5 mM [Ca$^{2+}$], was subtracted from Tg peak or maximal [Ca$^{2+}$], respectively.

**TIRF microscopy.** HEK293 were co-transfected with 6 μg STIM2.1-mcherry or STIM2.2-mcherry-pYRES and 2 μg Orai1-GFP-pMAX and seeded on 25-mm glass coverslips 24 h before measurement. Stacks were depleted by incubation with 1 μg Tg in 0 Ca$^{2+}$ Ringer solution for 15 min. A Leica AM TIRF MC system was used as in ref. 6. The TIRF focal plane was set to acceptor fluorescence and three sets of measurements were conducted 14–18 h later. The sequences of the siRNA are listed below. Knockdown efficiency and off-target effects were tested by qRT–PCR, where the mRNA level was normalized to TBP. Sequences of the used siRNA and qRT–PCR primers are listed in Supplementary Table 1.

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Expression and purification of the CAD domains. The cDNA coding for CAD domains of human STIM2.2 or STIM2.1 were amplified using the above mentioned pEX plasmids with primers including Rev and Hhc and Xma restriction sites and subcloned into a self-modified pET19b vector creating an N-terminal 6 His tag. Plasmids were expressed in E. coli BL21 Rosetta strain. Overnight cultures were inoculated in 2 x YT medium containing 0.4% glucose and on the next day diluted 1:20, grown to an OD600 of 0.5 before protein production was induced with 1 mM isopropyl-β-D-thiogalactoside for 6 h at 37°C. Lysis and protein purification were done as in ref. 42. After purification of the His-tagged domains, excess urea and imidazole were removed using illustra NAP-5 Columns (GE Healthcare), and protein was eluted in buffer containing 500 mM NaCl, 50 mM Tris and 0.1% Triton X-100, pH 7. Fractions of the protein were collected and protein concentration was determined using the BCA protein assay kit (Pierce).

Homology modelling and protein–protein docking analyses. Full details of STIM2 homology modelling and protein–protein docking analyses is given in Supplementary Tables 2–4, Supplementary Figs 8 and 9 and Supplementary Methods.

Surface plasmon resonance analysis. SPR spectroscopy was carried out in a BLItz X system. Briefly, monoclonal goat anti-GST serum (BIACORE, Freiburg, Germany) was immobilized on a CM5 research grade sensor chip (BIACORE) by amine coupling according to the manufacturer’s instructions. The chip was equilibrated with application buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 6.4 KCl, 2 mM MgCl2 and 0.005% F2O) at a flow rate of 20 μl/min−1. GST-CaM was bound to the immobilized antibodies in the measuring cell. Similarly, immobilized GST and the reference cell served as a negative control. Subsequently, solutions containing increasing concentrations of purified STIM2.1 and STIM2.2 CAD domains (7.5–750 nM) were passed over the chip in the presence of 2 mM Ca2+. Each protein application was followed by application of running buffer. The analysis was carried out employing the BIA evaluation software version 3.1 (BIACORE).

Peptide-spot-binding assay. Peptides encoding for STIM2 CAD domains were synthesized on acid-harden cellulose membranes, derivatized with a polyethylene glycol spacer as described43. Membranes were equilibrated in binding buffer (150 mM NaCl, 50 mM Tris/HCl (pH 7.5), 0.1% Triton X-100 and 1 mM CaCl2 or 4 mM EGTA) for 2 h at 4°C. 1C-labelled GST-CaM was added and incubated at 4°C overnight. The membrane was washed with binding buffer three times for 10 min each, dried at room temperature and subjected to phosphorimaging using a Typhoon-Trio imaging device (GE Healthcare). An equivalent peptide filter was calibrated with application buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 6.4 KCl, 2 mM MgCl2 and 0.005% F2O) at a flow rate of 20 μl/min−1. GST-CaM was bound to the immobilized antibodies in the measuring cell. Similarly, immobilized GST and the reference cell served as a negative control. Subsequently, solutions containing increasing concentrations of purified STIM2.1 and STIM2.2 CAD domains (7.5–750 nM) were passed over the chip in the presence of 2 mM Ca2+. Each protein application was followed by application of running buffer. The analysis was carried out employing the BIA evaluation software version 3.1 (BIACORE).

Calmodulin pull-down assay. The purified STIM2.1 and STIM2.2 CAD domains were incubated at a final concentration of 2.5 μM in total volume of 250 μl with 25 μl prewashed CaM-saharose beads (GE Healthcare) for 1 h at 4°C with overhead rotation. The binding buffer contained 25 mM Tris, pH 7.5, 150 mM NaCl and 1 mM CaCl2 or 1 mM EGTA. Unbound fraction was removed by washing three times with the corresponding buffer and bound fraction was eluted by boiling at 65 °C for 15 min with 2 x Laemmli buffer and finally analysed by electrophoresis. The western blot was probed with anti-His antibody (Cell Signaling) at 1:10,000 dilution. Uncropped versions of all blot and gel images are shown in Supplementary Fig. 10.

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**Author contributions**

A.-M.M., D.A. and B.A.N. conceived the project, designed the experiments and wrote the manuscript. A.-M.M. and D.A. performed the experiments and analysed the data. G.S. contributed the qRT–PCR analysis and gave technical support. P.-H.L. and V.H. undertook homology modelling and protein–protein docking analyses. M.J. designed peptide blots and performed radioactive experiments.

**Additional information**

**Supplementary Information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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