Proteomic mapping of ER–PM junctions identifies STIMATE as a regulator of Ca²⁺ influx

Ji Jing, Lian He, Aomin Sun, Ariel Quintana, Yuehe Ding, Guolin Ma, Peng Tan, Xiaowen Liang, Xiaolu Zheng, Liangyi Chen, Xiaodong Shi, Shenyan L. Zhang, Ling Zhong, Yun Huang, Meng-Qiu Dong, Cheryl L. Walker, Patrick G. Hogan, Youjun Wang and Yubin Zhou

Specialized junctional sites that connect the plasma membrane (PM) and endoplasmic reticulum (ER) play critical roles in controlling lipid metabolism and Ca²⁺ signalling. Store-operated Ca²⁺ entry mediated by dynamic STIM1–ORAI1 coupling represents a classical molecular event occurring at ER–PM junctions, but the protein composition and how previously unrecognized protein regulators facilitate this process remain ill-defined. Using a combination of spatially restricted biotin labelling in situ coupled with mass spectrometry and a secondary screen based on bimolecular fluorescence complementation, we mapped the proteome of intact ER–PM junctions in living cells without disrupting their architectural integrity. Our approaches led to the discovery of an ER-resident multi-transmembrane protein that we call STIMATE (STIM-activating enhancer, encoded by TMEM110) as a positive regulator of Ca²⁺ influx in vertebrates. STIMATE physically interacts with STIM1 to promote STIM1 conformational switch. Genetic depletion of STIMATE substantially reduces STIM1 puncta formation at ER–PM junctions and suppresses the Ca²⁺–NFAT signalling. Our findings enable further genetic studies to elucidate the function of STIMATE in normal physiology and disease, and set the stage to uncover more uncharted functions of hitherto underexplored ER–PM junctions.

ER–PM junctions are defined as spatially extended or small circular compartments in which the PM and ER membranes are stably separated at a distance of approximately 10–20 nm without direct membrane fusion. The broad significance of this structure has only recently begun to be appreciated, with emerging roles in lipid metabolism and Ca²⁺ homeostasis. Although ER–PM junctions were first observed over half a century ago, systematic dissection of this specialized subcompartment at the molecular level remains challenging owing to the lack of appropriate methods and convenient tools, which motivated us to explore a non-biased yet effective approach to obtain an integrated picture of ER–PM junctions. By taking advantage of an in situ protein labelling technique, we set out to map the proteome of ER–PM junctions that are dedicated to store-operated calcium entry (SOCE), a fundamental physiological process that is mediated by two protein families, STIM and ORAI (ref. 3). The ER-resident Ca²⁺ sensor protein STIM1, when fused to an engineered ascorbate peroxidase 2 (APEX2), enables proximity biotin labelling with least perturbation to the architecture of ER–PM junctions, thereby opening new avenues for capturing protein complexes situated at or in close proximity of STIM1 in living cells (Fig. 1a). At the heart of this in situ protein labelling technique is APEX2-catalysed conversion of biotin–phenol in the presence of H₂O₂ to phenoxyl radicals, which could attack electron-rich amino acids and covalently attach biotin tags to targeted proteins. Biotinylated proteins can be subsequently enriched by streptavidin beads and analysed by mass spectrometry. Notably, these radicals have very short lifetimes (<1 ms) with an estimated labelling radius of <20 nm (refs 5,9,10), thus matching the distance that separates ER and PM at junctional sites. One additional benefit of this approach is that it enables dynamic sampling of this specialized cellular compartment during the translocation of STIM1 from ER towards PM, thereby allowing us to compare protein complexes surrounding STIM1 before and after store depletion.

Following ER Ca²⁺ depletion elicited by thapsigargin, a blocker of the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA), total internal reflection fluorescence (TIRF) microscope imaging showed...
that EGFP-tagged STIM1–APEX2 formed puncta and co-localized tightly with biotinylated proteins, the latter of which were labelled by fluorophore-conjugated streptavidin (Fig. 1b). This process was dependent on H₂O₂ because biotinylation was not observed when omitting H₂O₂ in the reaction medium. The H₂O₂- and APEX2-dependent biotinylation of protein complexes surrounding STIM1...
was further confirmed by silver staining after affinity enrichment and independently by western blotting probed by streptavidin–HRP (Fig. 1c). As ORAI1 (relative molecular mass, 33,000 (M, 35K)) is a known binding partner of STIM1, we first analysed a gel slice corresponding to M, 25K–37K on the SDS–PAGE with mass spectrometry in our initial experiment and confirmed the presence of ORAI1 using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). In the same gel band, we repeatedly detected the gene product of TMEM110 (RefSeq ID: NM_198563), hereafter designated as STIMATE (for STIM-activating enhancer, Supplementary Fig. 1). In our subsequent LC–MS/MS analyses on all of the eluted products, we further identified a total of 73 potential STIM1 interactors, with 17 detected in both store-full and store-depleted HEK293 cells (Supplementary Tables 1 and 2). Most of them fell into the categories of ER– or PM-resident proteins, cytoskeletal components, and proteins functioning in intracellular membrane trafficking or post-translational modifications. Notably, 18% of them also appeared as strong hits identified in an independent genome-wide screen for regulators of nuclear factor of activated T-cells (NFAT; ref. 11), a transcriptional factor that is downstream of Ca\(^{2+}\) influx mediated by STIM–ORAI1 signalling. The prowess of this approach was further attested by the identification of EB1, SERCA2, ORAI1, voltage-dependent Ca\(^{2+}\) channels, extended synaptotagmin 1 and septins as candidate proteins that are in close proximity to STIM1 (Supplementary Tables 1 and 2). These proteins are known to either directly interact with STIM1 or reside in the ER–PM junctions.

Given the major interest in identifying protein regulators at ER–PM junctions that are directly involved in STIM1-dependent Ca\(^{2+}\) influx, we created a customized prey library of 38 genes representing most gene products (for example, STIMATE) identified under both store-full (resting) and store-depleted conditions, as well as candidate genes that have an annotated function in Ca\(^{2+}\) signalling or harbour predicted transmembrane domains (Supplementary Table 3). A secondary screen based on bimolecular fluorescence complementation (BiFC, Supplementary Fig. 1a–c) was performed to examine their potentials as STIM1 interactors either before or after thapsigargin-induced store depletion. Our assay is based on the reconstitution of an intact yellow fluorescent protein (YFP) from two complementary non-fluorescent fragments when they are trapped intracellularly when the last 74 amino acids were truncated (STIMATE\(_{1–246}\), Fig. 2a). Through a serial truncation study combined with site-directed mutagenesis, we further mapped a region (residues 241–KXRXRR246) that seems to be critical for keeping STIMATE localized within the ER. Substitution of these positively charged residues with glutamines (K or R→Q mutant) caused a large fraction of the ER-resident STIMATE\(_{1–250}\)–mCherry to exhibit a PM-like distribution (Fig. 2a). Next, we performed a chemical crosslinking experiment to examine the oligomeric state of STIMATE. As shown in Fig. 2c, addition of the membrane-permeable crosslinker DSS resulted in the appearance of protein bands corresponding to multimers of STIMATE on SDS–PAGE, suggesting that STIMATE could be assembled as oligomers in ER membrane. This result echoes the presence of a GXXXG transmembrane association motif\(^{22}\) in one of its predicted transmembrane segments (Supplementary Fig. 1d). The tendency of STIMATE to multimerize was further attested by the efficient restoration of YFP fluorescence when STIMATE was carboxy-terminally tagged with two complementary non-fluorescent YFP fragments in the BiFC assay (Supplementary Fig. 1b,c). The membrane topology of STIMATE was further determined by using a fluorescence protease protection assay\(^{23}\) with EGFP-STIM1 and STIM1–YFP as controls. We found that both termini of STIMATE face towards the cytosolic side of ER (Supplementary Fig. 1e–h).

We next examined whether STIMATE is located in the vicinity of the two main players of SOCE, STIM1 and ORA1. When expressed together with ORA1 or STIM1 in HEK293 cells, STIMATE co-localized tightly with STIM1 to ER but not with ORA1 at resting conditions (Fig. 2d), a finding that is further corroborated by results from an independent fluorescence resonance energy transfer (FRET) assay (Fig. 2e). Discernible FRET signals were detected only in HEK293 cells co-transfected with CFP–STIMATE and STIM1–YFP but not in cells coexpressing CFP–STIMATE and ORA1–YFP (Fig. 2e). When expressed alone or coexpressed with an ER
Figure 2 STIMATE is an ER-resident protein that co-localizes with STIM1. (a) Predicted domain architecture of STIMATE and confocal images of indicated STIMATE variants tagged with a C-terminal mCherry. STIMATE is predicted to contain 4–5 transmembrane segments (see Supplementary Fig. 1). TM, predicted transmembrane segment. D/E: a negatively charged region in the C terminus of STIMATE (STIMATE-CT). K/R: a polybasic domain in STIMATE-CT. Blue, nuclear staining with Hoechst 33342. Scale bar, 10 μm. (b) Confocal sections (at the footprint or the middle plane) of HEK293 cells coexpressing EGFP–STIMATE (green) and the ER marker mCherry–Sec61β (red). Nuclei were labelled in blue by Hoechst 33342. The insets show the regions outlined by dashed lines at higher magnification. Scale bar, 10 μm. (c) Crosslinked FLAG–STIMATE resolved on SDS–PAGE. FLAG-tagged STIMATE was transiently expressed in HEK293 cells and subjected to chemical crosslinking with 0–250 μM DSS (a membrane-permeable crosslinker) on ice for 5 min. The arrowheads indicate the appearance of FLAG–STIMATE oligomers on crosslinking. (d) Confocal images of EGFP–STIMATE in HEK293 cells coexpressing mCherry–ORAI1 or mCherry–STIMATE without store depletion. Blue, nuclear staining with Hoechst 33342. Scale bar, 10 μm. (e) Quantification of FRET signals in HEK293 cells coexpressing CFP–STIMATE with ORAI1–YFP (n = 5 cells) or with STIM1–YFP (n = 10 cells) pooled across three independent experiments. Error bars denote s.e.m. (f) Representative TIRF images of an HEK293 cell expressing STIMATE–YFP before and after store depletion induced by 1 μM thapsigargin (TG). Scale bar, 10 μm. (g) Representative TIRF images of an HEK293 cell coexpressing EGFP–STIM1 (green) and mCherry–STIMATE (red) before and after store depletion induced by 1 μM thapsigargin. The single-channel images are shown in greyscale. The plot on the right shows the quantification of STIM1–STIMATE co-localization under TIRF microscopy by Pearson's correlation coefficient. Error bars denote s.e.m. for n = 6 cells pooled across two independent experiments. Scale bar, 10 μm. Unprocessed original scans of blots/gels are shown in Supplementary Fig. 6.
Figure 3 STIMATE facilitates efficient formation of STIM1 puncta at ER–PM junctions. (a) Confocal images at the footprint of STIM1–YFP + ORAI1–CFP HEK293 cells transiently transfected with mCherry-STIMATE. STIM1, ORAI1 and STIMATE are artificially coloured in green, red and grey, respectively, to aid visualization. Store depletion was triggered by 1 μm thapsigargin (TG). Scale bar, 10 μm. (b) Quantification of STIM1 cluster size in thapsigargin-stimulated cells treated as in a. The rectangle in the box plot shows the distribution of data between the first and third quartiles. The segment inside the rectangle shows the median, whereas the whiskers above and below the box mark the minimum and maximum. ***P < 0.001 (n=15 cells pooled across three independent experiments; paired Student’s t-test). Error bars denote s.e.m. for n=6 cells pooled across three independent experiments. (d) Representative TIRF images of STIM1 acquired 0, 90 or 300 s after depletion of ER Ca²⁺ stores with 1 μM thapsigargin in normal or STIMATE-KO HEK293 stable cells. Scale bar, 10 μm. (e) Time course of EGFP–STIM1 puncta formation (upper left panel) and quantification of STIM1 puncta size (upper right panel) and their distribution profiles (lower panel). Store depletion was induced by 1 μM thapsigargin. ***P < 0.001 (compared with control; paired Student’s t-test). Error bars denote s.e.m. for n=5–10 cells. (f) Electron micrographs of HRP–STIM1 in normal or STIMATE-KO HEK293 cells before and after store depletion triggered by 1 μM thapsigargin. Blue arrowheads, HRP–STIM1 staining in non-cortical ER that is separated <20 nm from PM. The bar graph on the right represents the quantification of HRP–STIM1 distribution in different pools of the ER (average of 5–10 cells). Scale bar, 2,000 nm. N, nucleus.

depletion. In contrast, the other cells in the same field without STIMATE overexpression showed an even distribution of STIM1 at the footprint of HEK293 cells (Fig. 3a). On store depletion, STIMATE-expressing cells formed significantly larger STIM1 puncta (Fig. 3b). Consistent with the pre-formation of STIM1 puncta before store depletion, overexpression of mCherry–STIMATE also elicited
constitutive Ca\(^{2+}\) influx in these stably expressing cells when we switched the extracellular Ca\(^{2+}\) concentration from 0 to 1 mM (Fig. 3c). The Ca\(^{2+}\) flux could be reversed by withdrawing Ca\(^{2+}\) or by adding 2-APB, a pharmacological tool that is widely used at high concentrations (~50–100 mM) to block SOCE.

These findings led us to propose that STIMATE could modulate STIM1 puncta formation at ER–PM junctions. Indeed, genetic disruption of STIMATE in HEK293 (STIMATE-KO) cells induced a substantial reduction in the amounts of STIM1 puncta at ER–PM junctions (Fig. 3d and Supplementary Video 1). Compared with thapsigargin-stimulated normal HEK293 cells, the fluorescence intensity of EGFP–STIMATE-KO cells dropped by almost 70%. STIMATE knockout appreciably delayed the formation of STIM1 puncta, and resulted in a significant reduction in the puncta size at ER–PM junctions (Fig. 3e). An appreciable (~10–15%), but less pronounced, reduction in puncta formation was also observed in STIMATE-KO cells expressing the STIM1 gain-of-function mutants D76A and L258G (Supplementary Fig. 5a–d). Subsequent scrutiny under high-resolution electron microscopy further revealed that fewer STIM1 proteins migrated into cortical ER (cER, Fig. 3f and Supplementary Fig. 5e), a specialized ER network that lies immediately beneath the PM (~20 ~ 30 nm). In aggregate, these results indicate that STIMATE is required to promote efficient STIM1 clustering at ER–PM junctions, which might be achieved through modulating cER accumulation or modulating STIM1 activation as described below.

To examine the effect of STIMATE depletion on cER, we compared the cER accumulation in native and STIMATE-KO HEK293 cells. We used the genetically encoded fluorescent cER marker, MAPPER (membrane-attached peripheral ER), as readout because it has been recently shown to selectively label cER and co-localize tightly with activated STIM1 puncta at ER–PM junctions\(^{20}\). We noted that depletion of STIMATE caused approximately 8–10% decrease in the intensity or density of cER in HEK293 cells (Supplementary Fig. 5f–h). To rule out the possibility that the constitutive localization of MAPPER at ER–PM junctions might cause bias in our analysis on cER, we created an optogenetic tool, LiMETER (light-inducible membrane-tethered peripheral ER, Fig. 4 and Supplementary Video 2), to reversibly label cER. The ER luminal domain of LiMETER is composed of a signal peptide and the single transmembrane domain derived from STIMATE, with GFP placed in between as a reporter. The cytoplasmic region of LiMETER contains a flexible linker and a genetically encoded lightswitch LOV2 domain (light oxygen voltage-sensing domain, residues 404–546).
**Figure 5** STIMATE interacts with STIM1 cytosolic fragments and promotes STIM1 conformational switch. (a) GFP–STIM1 coimmunoprecipitated with mCherry-tagged STIMATE but not mCherry (control). (b) FLAG–STIMATE coimmunoprecipitated with mCherry–STIMATE and the three indicated STIM1–CT fragments, but not with the minimal ORAI-activating domain CAD or SOAR. The domain architecture of STIM1 and design of truncated variants are shown at the top. (c) FRET signals measured in HEK293 cells co-transfected with the indicated donor-acceptor pairs without store depletion. YFP was C-terminally tagged to STIM1–342 and STIM1–310, and N-terminally fused with STIM1–CT (233–685) and SOAR or CAD. Error bars denote s.e.m. for n = 15, 18, 20 or 17 cells, respectively (from left to right) pooled across three independent experiments. cEF, canonical EF-hand; hEF, hidden EF-hand; SAM, sterical alpha-motif; TM, transmembrane domain; SOAR/CAD, STIM-Orai activating region; SnPn, serine and proline-rich domain; K, polybasic C-tail. (d) In vitro binding of recombinant STIM1–CT and STIM1–CC1 to recombinant GST–STIMATE–CT proteins immobilized on glutathione (GS4B) resin. GST was used as a negative control. (e) Surface plasmon resonance measurements of the interaction between STIM1–CC1 and STIMATE–CT in vitro. Sensorgrams monitor the binding of STIMATE–CT (with concentrations labelled on the right) to immobilized STIM1–CC1 (with an N-terminal CGG linker to aid thiol coupling to the CM3 sensor chip). Inset: the peak values for each sensorgram were collected and a dose–response binding isotherm was created to obtain an apparent dissociation constant of 8.9 μM. One set of data representing three reproducible, independent experiments is shown. (f) FRET signals between STIM1–1–310–CFP (donor) and YFP–SOAR (acceptor) in HEK293 cells coexpressing mCherry (control) or mCherry–STIMATE (red, M. One set of data representing three reproducible, independent experiments). Ionomycin (0.4 μM) was added to deplete ER Ca2+ stores. Left, time course. Right, bar graph of resting FRET signals. *P < 0.05; **P < 0.01 (paired Student’s t-test). Error bars denote s.e.m. (g,h) FRET signals in HEK293 cells stably expressing ORAI1–CFP (donor) and YFP–STIM1 (acceptor) with concentrations labelled on the right) to immobilized STIM1–CC1 (with an N-terminal CGG linker to aid thiol coupling to the CM3 sensor chip). Inset: the peak values for each sensorgram were collected and a dose–response binding isotherm was created to obtain an apparent dissociation constant of 8.9 μM. One set of data representing three reproducible, independent experiments is shown. (g) STIM1–1–310+STIMATE (red, M. One set of data representing three reproducible, independent experiments). Ionomycin (0.4 μM) was added to deplete ER Ca2+ stores. Left, time course. Right, bar graph of resting FRET signals. *P < 0.05; **P < 0.01 (paired Student’s t-test). Error bars denote s.e.m. (h) FRET signals in HEK293 cells stably expressing ORAI1–CFP (donor) and YFP–STIM1 (acceptor) with concentrations labelled on the right) to immobilized STIM1–CC1 (with an N-terminal CGG linker to aid thiol coupling to the CM3 sensor chip). Inset: the peak values for each sensorgram were collected and a dose–response binding isotherm was created to obtain an apparent dissociation constant of 8.9 μM. One set of data representing three reproducible, independent experiments is shown. (g,h) FRET signals in HEK293 cells stably expressing ORAI1–CFP (donor) and YFP–STIM1 (acceptor) with concentrations labelled on the right) to immobilized STIM1–CC1 (with an N-terminal CGG linker to aid thiol coupling to the CM3 sensor chip). Inset: the peak values for each sensorgram were collected and a dose–response binding isotherm was created to obtain an apparent dissociation constant of 8.9 μM. One set of data representing three reproducible, independent experiments is shown. (g,h) FRET signals in HEK293 cells stably expressing ORAI1–CFP (donor) and YFP–STIM1 (acceptor) with concentrations labelled on the right) to immobilized STIM1–CC1 (with an N-terminal CGG linker to aid thiol coupling to the CM3 sensor chip). Inset: the peak values for each sensorgram were collected and a dose–response binding isotherm was created to obtain an apparent dissociation constant of 8.9 μM. One set of data representing three reproducible, independent experiments is shown.
derived from *Avena sativa* phototropin 1 (refs 26,27), followed by a C-terminal PM-targeting polybasic tail isolated from the small G protein Rit28 (Fig. 4a). In the dark, the α-helix docks to the LOV2 domain and cages the polybasic tail to prevent its interaction with negatively charged PM-resident phosphoinositides. On blue light stimulation, photoexcitation generates a covalent adduct between a cysteine residue (Cys 450) and the flavin cofactor in LOV2, and subsequently promotes the undocking and unwinding of the α-helix26,27, thereby exposing the Rit polybasic C-tail to enable translocation of the protein towards PM to form puncta-like structures28. As a result, LiMETER underwent photo-inducible accumulation at ER–PM junctions to specifically label cER (Fig. 4 and Supplementary Video 2). Notably, this process can be reversibly repeated with multiple light–dark cycles without significant loss in the magnitude of response (Fig. 4d). This unique tool enables us to quantitatively examine the effect of STIMATE deactivation on the dynamics of cER accumulation at defined spatiotemporal resolution. When compared with native HEK293 cells, we observed 10–12% decrease in the rate and extent of LiMETER accumulation at ER–PM junctions in STIMATE-KO HEK293 cells after blue light illumination (Fig. 4c,d). Thus, by using either MAPPER or LiMETER as a cER marker, we found that genetic depletion of STIMATE noticeably affects cER accumulation in HEK293 cells. Nonetheless, the ~10% decrease in the efficiency of cER accumulation could not fully explain the more pronounced effect (~70%) on STIM1 puncta formation in STIMATE-depleted cells (Fig. 3d,e). This prompted us to further explore alternative mechanisms by focusing on the initial activation steps of STIM1.

Results from both the BiFc (Supplementary Fig. 1a–c) and FRET (Fig. 2e) assays suggest a possible interaction between STIMATE and STIM1 in HEK293 cells. We further confirmed this by using communoprecipitation (Fig. 5a). To investigate how STIMATE modulates STIM1 actions, we applied a combination of communoprecipitation (Fig. 5b) and FRET (Fig. 5c) methods to map the domain(s) within STIM1 that mediate its interaction with STIMATE. We narrowed down the minimal STIMATE-interacting domain to the juxtamembrane coiled coil region of STIM1 (STIM1-CC1, residues 233–343). In both assays, the minimal ORAI1-activating domain of STIM1 (CAD/SOAR; refs 29,30) showed negligible or no discernible interaction with STIMATE. As the longest cytosolic fragment of STIMATE protein is its C-terminal region (STIMATE-CT, residues 214–294, Fig. 2a and Supplementary Fig. 1d), we further expressed STIMATE-CT in bacteria and purified the recombinant protein to examine whether it would directly interact with STIM1 cytosolic domains. GST-tagged STIMATE-CT immobilized on G4S4B resin was able to pull down both the entire STIM1 cytoplasmic domain (233–685) and STIM1-CC1 (Fig. 5d). The dissociation constant of the interaction between STIMATE-CT and STIM1-CC1 was further determined to be 8.9 ± 0.7 μM (Fig. 5e). Overall, these studies clearly demonstrated a direct physical contact between STIMATE and STIM1 in vitro.

After store depletion, STIM1 is activated through a conformational switch mechanism by undocking CAD/SOAR from CC1, thus projecting CAD/SOAR towards the PM to engage and gate ORAI1 channels29. The observation of a direct interaction between STIMATE-CT and STIM1-CC1 raised the possibility that STIMATE might promote STIM1 conformational switch through disrupting the intramolecular trapping mediated by CC1–SOAR association32. To test this, we developed a two-component FRET assay by fusing CFP or YFP to STIM11–310 and SOAR, respectively. This assay enabled us to monitor CC1–SOAR interaction in real time, and more importantly, to examine the effect of STIMATE on the STIM1 conformational switch in living cells32. Under resting conditions, YFP–SOAR was tightly docked towards the ER-resident STIM11–310 and showed a high resting FRET signal (Fig. 5f). On ionomycin stimulation, YFP–SOAR undocked from CC1 and thus caused a profound decrease of the FRET signal. The coexpression of STIMATE in this assay significantly reduced the resting FRET signal (Fig. 5f), implying that STIMATE could at least partially release STIM1 autoinhibition possibly by weakening CC1–SOAR association at rest. STIMATE-CT exhibited a similar effect but was much less potent, probably because this process requires anchoring of the STIMATE C-tail in the vicinity of the ER membrane or it involves coordinated actions of the C-tail with other regions of STIMATE. The consequence of STIMATE-mediated action on STIM1 was further reflected in enhanced FRET signals between CFP-STIM1 and YFP–STIM1 (Fig. 5g) or STIM1–YFP and ORAI1–CFP at rest (Fig. 5h), and culminated in constitutive Ca2+ influx (Fig. 3c). Clearly, our data provide compelling evidence to support a model in which STIMATE interacts with the juxtamembrane CC1 region of STIM1 to perturb the CC1–SOAR association that is the basis for STIM1 autoinhibition32, thereby shifting the equilibrium towards activated states to promote the conformational switch and subsequent translocation of STIM1 towards ER–PM junctions.

In summary, we have applied a non-disruptive biotin labelling approach to capture protein complexes in close proximity to STIM1 at ER–PM junctions in living cells. Our findings not only afford an initial view on the protein composition of intact ER–PM junctions under physiological conditions, but also provide a framework for future dissection of their roles in cellular signalling, human health and disease. In particular, our study has established an irreplaceable role of the previously unrecognized ER-resident protein STIMATE in modulating STIM1-dependent Ca2+ signalling at ER–PM junctions. Major questions that warrant further investigation include: how STIMATE chaperographs with other components at ER–PM junctions to coordinate Ca2+ signalling and other cellular events; and how STIMATE itself contributes to the formation and maintenance of ER–PM junctions. □

**METHODS**

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

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

**ACKNOWLEDGEMENTS**

We are grateful to R. Lewis at Stanford University for the HRP–STIM1, mCherry–STIM1, and mCherry–CAD constructs. We thank J. Liou at University of Texas Southwestern Medical Center for sharing with us the MAPPERs construct, and Z. Songyang at Baylor College of Medicine for the BiFc-related constructs. We thank M. Hooke at Texas A&M University for access to the Biacore 3000, D. Liu at Baylor College of Medicine for access to the Cell Based Assay Screening Facility and advice on BiFc, and R. Payne at Texas A&M University for technical support in electron microscopy studies. This work was supported by National Institutes of Health grants (R01 GM112003 to Y.Z., R01 AI084167, R01 CA143811 to C.L.W., and R01 GM110397 to P.G.H.), a Special Fellow Award from the Leukemia & Lymphoma Society (LLS 3013-12 to Y.Z.), a Robert A. Welch Endowed Chair in Chemistry (BE-0023) to C.L.W., the China Scholarship Council (to J.J.), the National Natural Science Foundation of China (NSFC 31471279 to Y.W. and NSFC-81322020 to L.C.), the Recruitment Program for Young Professionals of China (to Y.W.),
the Program for New Century Excellent Talents in University (NCET-13-0061 to Y.W.), the American Heart Association SDG (13SDG17200006 to S.L.Z.), a Cancer Prevention Research Institute of Texas grant (to Y.H.), and by an allocation from the Texas A&M University Health Science Center Startup Fund (to Y.Z.).

AUTHOR CONTRIBUTIONS
Y.Z. and Y.W. supervised and coordinated the study. L.H., J.J., Y.Z. and Y.Y. designed and generated all the plasmid constructs. L.H. performed the BiFC assays. J.J., P.T. and L.H. generated the knockout cell lines. L.H., Y.D. and M.-Q.D. prepared the proteomic samples and performed the mass spectrometry analyses. G.M., J.J., X.L. and Y.Z. developed the in vitro assays, and carried out the experiments with assistance from L.H., P.T. and Y.H. A.S., J.J., Y.W. and S.L.Z. performed the Ca\textsuperscript{2+} influx assay. J.J., A.S., A.Q., L.H., X.Z., L.C., L.Z. and Y.W. performed all the fluorescence imaging and other cell-based experiments. X.S. contributed to the synthesis of biotin–pheno. Y.Z., J.J., Y.D., L.H., A.S., G.M., Y.W. and M.-Q.D. analysed data, with input from the other authors. P.G.H., Y.H. and C.L.W. provided intellectual inputs to the manuscript. J.J., Y.W. and Y.Z. wrote the manuscript.

COMPEING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3234
Reprints and permissions information is available online at www.nature.com/reprints

1. Carrasco, S. & Meyer, T. STIM proteins and the endoplasmic reticulum-plasma membrane junctions. Annu. Rev. Biochem. 80, 973–1000 (2011).
2. Elbaz, Y. & Schuldiner, M. Staying in touch: the molecular era of organelle contact sites. Trends Biochem. Sci. 36, 616–623 (2011).
3. Hogan, P. G., Lewis, R. S. & Rao, A. Molecular basis of calcium signaling in lymphocytes: STIM and Orai. Annu. Rev. Immunol. 28, 491–533 (2010).
4. Stefan, C. J., Manford, A. G. & Emr, S. D. ER-PM connections: sites of information transfer and inter-organelle communication. Curr. Opin. Cell Biol. 25, 434–442 (2013).
5. Rhee, H. W. et al. Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. Science 336, 1328–1331 (2013).
6. Lam, S. S. et al. Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat. Methods 12, 51–54 (2015).
7. Kerppola, T. K. Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. Annu. Rev. Biophys. 37, 465–487 (2008).
8. Porter, K. R. & Palade, G. E. Studies on the endoplasmic reticulum. Ill. Its form and distribution in striated muscle cells. J. Biophys. Biochem. Cytol. 3, 269–300 (1957).
9. Mayer, G. & Bendayan, M. Biotinyl-tyramide: a novel approach for electron microscopic immunocytochemistry. J. Histochem. Cytochem. 45, 1449–1454 (1997).
10. Bendayan, M. Tech.Sight. Worth its weight in gold. Science 291, 1363–1365 (2001).
11. Sharma, S. et al. An siRNA screen for NFAT activation identifies septins as coordinators of store-operated Ca\textsuperscript{2+} entry. Nature 499, 238–242 (2013).
12. Min, S. W., Chang, W. P. & Sudhof, T. C. E-Syts, a family of membranous Ca\textsuperscript{2+}-sensor proteins with multiple C2 domains. Proc. Natl Acad. Sci. USA 104, 3823–3828 (2007).
13. Manjarres, I. M., Rodriguez-Garcia, A., Alonso, M. T. & Garcia-Sancho, J. The sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) is the third element in capacitative calcium entry. Cell Calcium 47, 412–418 (2010).
14. Wang, Y. et al. The calcium store sensor, STIM1, reciprocally controls Orai and Ca\textsuperscript{2+} channels. Science 330, 105–109 (2010).
15. Park, C. Y., Shcheglovitov, A. & Dolmetsch, R. The CRAC channel activator STIM1 binds and inhibits L-type voltage-gated calcium channels. Science 330, 101–105 (2010).
16. Grigoriev, I. et al. STIM1 is a MT-plus-end-tracking protein involved in remodeling of the ER. Curr. Biol. 18, 177–182 (2008).
17. Soboloff, J., Rothsberg, B. S., Madesh, M. & Gill, D. L. STIM proteins: dynamic calcium signal transducers. Nat. Rev. Mol. Cell Biol. 13, 549–565 (2012).
18. Giordano, F. et al. P(4,5)P(2)-dependent and Ca\textsuperscript{2+}-regulated ER-PM interactions mediated by the extended synaptotagmins. Cell 153, 1494–1509 (2013).
19. Tabb, D. L., McDonald, W. H. & Yates, J. R. 3rd DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. J. Proteome Res. 1, 21–26 (2002).
20. Chang, C. L. et al. Feedback regulation of receptor-induced Ca\textsuperscript{2+} signaling mediated by E-Syt1 and Nir2 at endoplasmic reticulum-plasma membrane junctions. Cell Rep. 5, 813–825 (2013).
21. Maleth, J., Choi, S., Muallem, S. & Ahuja, M. Translocation between PI(4,5)P\textsubscript{2}-rich and PI(4,5)P\textsubscript{2}-poor and PI(4,5)P\textsubscript{2}-rich microdomains during store depletion determines STIM1 conformation and Orai1 gating. Nat. Commun. 5, 5843 (2014).
22. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823 (2013).
23. Zurek, N., Sparks, L. & Voeltz, G. Reticulon short hairpin transmembrane domains are used to shape ER tubules. Traffic 12, 28–41 (2011).
24. Russ, W. P. & Engelman, D. M. The GoGxG motif: a framework for transmembrane helix-helix association. J. Mol. Biol. 296, 911–919 (2000).
25. Lorenz, H., Hailey, D. W., Wunder, C. & Lippincott-Schwartz, J. The fluorescence protease protection (TPP) assay to determine protein localization and membrane topology. Nat. Protoc. 1, 276–279 (2006).
26. Wu, Y. I. et al. A genetically encoded photoactivatable Rac controls the motility of living cells. Nature 461, 104–108 (2009).
27. Harper, S. M., Neil, L. C. & Gardner, K. H. Structural basis of a phototropin light switch. Science 301, 1541–1544 (2003).
28. Heo, W. D. et al. PI(3,4,5)P\textsubscript{3} and PI(4,5)P\textsubscript{2} lipids target proteins with polybasic clusters to the plasma membrane. Science 314, 1458–1461 (2006).
29. Park, C. Y. et al. STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. Cell 136, 876–890 (2009).
30. Yuan, J. P. et al. SOAR and the polybasic STIM1 domains gate and regulate Orai channels. Nat. Cell Biol. 11, 337–343 (2009).
31. Zhou, Y. et al. Initial activation of STIM1, the regulator of store-operated calcium entry. Nat. Struct. Mol. Biol. 20, 973–981 (2013).
32. Ma, G. et al. Inside-out Ca\textsuperscript{2+} signaling prompted by STIM1 conformational switch. Nat. Commun. 6, 7826 (2015).
**METHODS**

### Chemicals.
Tris(2-carboxyethyl)phosphine and disuccinimidyl suberate were obtained from Pierce. All other reagents were from Sigma Aldrich unless otherwise indicated. Biotin–phenol was initially obtained as a gift from A. Ting at Massachusetts Institute of Technology and later synthesized by following the reported synthesis scheme.

### Antibodies.
Streptavidin–DyLight594 (catalogue no. 21842, 1:2,000 for immunofluorescence staining) and streptavidin–HRP (N100, 1:4,000 for western blotting (WB)) were purchased from Thermo Scientific. Mouse monoclonal anti-FLAG antibody (clone number M2, catalogue no. F3165, 1:3,000 WB, 1:25 μg per 100 μg of total protein for immunoprecipitation (IP)) was purchased from Sigma. The rabbit polyclonal anti-GFP antibody (sc-8334, 1:1,000 WB, 0.5 μg per 100 μg of total protein for IP) was obtained from Santa Cruz Biotechnology. The mouse monoclonal and rabbit polyclonal anti-mCherry antibodies were obtained from Clontech (clone number 1C51, catalogue no. 632543, 1:1,000 WB) and Novus Biologicals (NPB2-25157, 1:2,000 WB), respectively. Mouse anti-rabbit IgG (1:1,000 WB, 0.5 μg per 100 μg of total protein for IP) was obtained from Jackson ImmunoResearch Laboratories.

### Cell lines.
Cell lines including HEK293 (CRL-1573), HeLa (CCL-2) and COS-7 (CRL-1615) were purchased from American Type Culture Collection (ATCC). The cell lines were propagated in short tandem repeats (STR) and confirmed to be STR negative. Cells stably expressing STIM1, ORAI1 or STIM1–ORAI1 were subsequently generated on the basis of the aforementioned cell lines after genotyping or puromycin selection. All of the cell lines were free of mycoplasma contamination.

### Plasmids.
The cDNA clone encoding human STIM1 was purchased from DNAsu Plasmid Repository. GFP–STIM1, STIM1–GFP and FLAG–STIM1 were generated through gateway cloning by using the vectors pcDNA-DEST54, pcDNA-DEST47 (Life Technologies), and a customized FLAG-tagged destination vector. STIM1–YFP and STIM1–CFP were made by inserting STIM1 between Nhel–BamHI sites of pEYFP-N1 or pECFP-N1 (ClonTech). mCherry–STIM1 or CFP–STIM1 was made by inserting amplified fragments into the pcDNA3.1(+) vector. mCherry-tagged STIM1 truncation variants were made by ligation into pmCherry-N1 between Nhel–BamHI sites. STIM1 mutant constructs were subsequently made by using the QuickChange Lightning Multi site-directed mutagenesis kit (Agilent). Full-length cDNA of human STIM1 was subcloned into pmCMV-XL5 (Origene) with the insertion of EGFP or YFP between two additional NarI sites introduced immediately after residue Asn. For STIM1–YFP constructs, human STIM1 was inserted into pEGFP-N1 between XhoI and BamHI. pCDNA3.1(+)–mCherry–ORAI1 was made by sequential insertion of mCherry and human ORAI1. The ER localising plasmid pDsRed2-ER was obtained from Clontech. mCherry–Sec61ER was obtained from the pEGFP-N1 between XhoI and BamHI. pcDNA3.1(+)–mCherry–CFP–ORAI1 was made by inserting amplified fragments into the pcDNA3.1(+) vector. mCherry–CFP–ORAI1 was made by inserting amplified fragments into the pcDNA3.1(+) vector.

### Proteomic mapping of ER–PM junction in HEK293 cells.
A step-by-step protocol describing GFP–STIM1–APEX2–mediated in situ biotinylation and further proteomic studies on ER–PM junctions can be found at Nature Protocol Exchange (http://dx.doi.org/10.1038/nprot.2015.072).

### Bioluminescence measurements. Intracellular [Ca2+] was measured with Fura-2 AM as previously described.

### Fluorescence resonance energy transfer measurements.
Intracellular [Ca2+]–free solution was used to keep cells healthy. Emission fluorescence at 509 nm generated by 340-nm excitation light at 509 nm was measured with a HTS sampler (BD Biosciences) through the Cell-Based Assay Screening Service facility at Baylor College of Medicine. The percentage of YFP–positive cells was calculated by the software FlowJo.

### Single-cell intracellular Ca2+ measurements.
Intracellular [Ca2+] was measured with Fura-2 AM as previously described.

### Fluorescence resonance energy transfer measurements.
The same system used in Ca2+ measurements was used to measure the apparent FRET efficiency, Eapp, using the following formula: E = F0/F0 + F − F − F0/F0. Where F0 represents measured bleed-through
of CFP into the FRET filter (0.84), and $F_{c}\text{/}D_{r}$ represents measured bleed-through of YFP through the FRET filter (0.13). Second, to reduce variations caused by differences in expression levels, FRET, values were further normalized against donor fluorescence ($F_{D}\text{/}D_{D}$) to generate N-FRET (normalized FRET) signal. Third, $E_{C}$ was calculated using the following equation: $E_{C} = \frac{F_{D}}{F_{D} + F_{C}}$, where $G = 0.59$ is the system-dependent factor. A fluorescence probe, YFP-STIM1-D76G–CFP, was expressed in HEK293 cells to examine the relative expression level of CFP-tagged protein to YFP-tagged ones. This construct was used as a negative control in our FRET experiments because the donor (CFP) and the acceptor (YFP) were separated by EMR membrane with a distance of >10 nm (ref. 1). To minimize fluctuations in $E_{C}$ caused by variations of relative expression levels of donor protein to acceptor protein, cells were cultured with a $F_{D}/F_{C}$ ratio falling between 0.7 1.4. These values were used for data analysis. All fluorescence images were collected and processed with MetaFlour software (Molecular Devices), and the resulting data were further analyzed with Matlab R2012b software and plotted with Prism5 software. Representative traces of at least three independent experiments are shown as mean ± s.e.m.

**NFAT-dependent luciferase assay.** HEK293 cells were cultured in DMEM as described above in 24-well plates. After reaching 40 50% confluence, siRNA oligonucleotides were transfected using DharmaFECT (GE Dharmacon) for 48h before further transfection of NFAT–luciferase reporter gene pGL3.40 [luc2P/NFAT-RE/Hygro] (Promega). The Renilla luciferase gene (pRL-TK) was co-transfected as a control for counting transfected cells and normalizing the luminescence signals. HEK293 cells were treated with phosphor 12-myristate 13-acetate (PMA; 1μM) and thapsigargin (1μM) for 8h. Three duplicates were used for each transfection. Cells were then collected and lysed by following the manufacturer’s protocol. Luciferase activity was assayed using the Dual Luciferase Reporter Assay System (Promega) on a Biotek Synergy2 luminescence microplate reader. The ratio of firefly to Renilla luciferase activity was plotted for HEK293 cells. All of the data were normalized against the control group. For the rescue group, after 48h treatment with siRNA, HEK293 luciferase was further transfected with the STIMATE-siRNA-resistant variant of mCherry-STIMATE, along with the NFAT-luciferase reporter genes and pRL-TK.

**DSS-mediated crosslinking in HEK293 cells.** HEK293 cells were plated in 6- well plates and maintained at 37°C and 5% CO2 for 24h before transfection. After transient transfection with FLAG-tagged STIMATE, cells were washed three times with ice-cold PBS (pH 8.0) and treated with 0, 50, 100 and 250 μM of freshly prepared DSS. The mixture was incubated on ice for 1 5min and the reaction was stopped by incubating the cells with a quenching solution containing 50mM Tris-HCl at pH 8.0 for 15min at room temperature. The cells were then lysed by lysis buffer (20mM HEPEs pH 7.4, 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate and 1mM β-glycero-phosphate), mixed with an equal volume of 2× sample loading buffer, heated at 42°C for 10min (to avoid aggregation), and subjected to electrophoresis on 4–12% precast gel (Bio-Rad). After transferring the lysates to the polyvinylidene fluoride (PVDF) membrane, it was Immunoblotted with monoclonal mouse anti-FLAG M2 antibody (1:3,000, Sigma).

**Confocal imaging and total internal reflection fluorescence (TIRF) microscopy.** Cell lines used for imaging include HEK293, HeLa and COS-7 cells lysates to the polyvinylidene fluoride (PVDF) membrane, it was immunoblotted with the indicated antibodies and magnetic A or G beads (Thermo Scientific) overnight at 4°C. The beads were pelleted and washed with lysis buffer, in the presence of protease inhibitors 10 times and were then heated in SDS loading buffer for 10min at 42°C before resolving on SDS–PAGE. The cell lysates were separated by 4 15% gels (Bio-Rad), transferred to PVDF membranes and probed with the indicated antibodies.

**BiAcore surface plasmon resonance (SPR) measurements.** SPR-based Biacore experiments were performed at 25°C on a Biacore 3000 instrument (GE Healthcare Bio-Sciences AB). The immobilized STIM1-CC1 sensor surface was prepared by ligand thiol coupling through the single cysteine residue at the N terminus of CCG-CC1 (ref. 31). The sensor chip CM3 and reagents for thiol coupling were purchased from GE Healthcare. The immobilization was performed at a 5 μL min−1 flow rate with PBS (10mM sodium phosphate pH 7.4, 150mM NaCl). The flow cell was activated by 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 0.1 M N-hydroxysuccinimide followed by washing with 80mL of 2-(2-pyridyldithio)ethylamine hydrochloride (GE Healthcare) in 0.1 M sodium borate (pH 8.5) to introduce reactive disulphide groups on the surface. Thirty microlitres of STIM1-CC1 (10μg mL−1 in 10mM sodium acetate pH 5.5) was injected to the activated surface and then blocked with 20μL of cysteine/NaCl. Approximately 550 RU (response unit) of STIM1-CC1 was immobilized. A reference flow cell was prepared with the same activation and blocking in steps but without any protein coupled. The binding study was performed at 50μL min−1 flow rate using TBS (20mM Tris, 150mM NaCl, pH 7.5) as running buffer. After each injection of STIM1-CC1 (twofold serial dilution in running buffer), the CC1 surface was regenerated by injecting 10mM glycine (pH 1.7) for 30s to remove bound STIM1-CC1. Background-corrected sensorgrams were collected for data analysis.

**Statistical analyses.** Unless otherwise noted, quantitative data are expressed as the mean and standard error of the mean (s.e.m.). Statistical significance was determined with paired Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001, when compared with control or WT.
33. Wu, M. M., Buchanan, J., Luik, R. M. & Lewis, R. S. Ca\textsuperscript{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. J. Cell Biol. 174, 803–813 (2006).

34. Lee, O. H. et al. Genome-wide YFP fluorescence complementation screen identifies new regulators for telomere signaling in human cells. Mol. Cell. Proteom. 10, M110001628 (2011).

35. Zhou, Y. et al. STIM1 gates the store-operated calcium channel ORAI1 in vitro. Nat. Struct. Mol. Biol. 17, 112–116 (2010).

36. Wang, Y. et al. STIM protein coupling in the activation of Orai channels. Proc. Natl Acad. Sci. USA 106, 7391–7396 (2009).

37. Zhou, Y., Ramachandran, S., Oh-Hora, M., Rao, A. & Hogan, P. G. Pore architecture of the ORAI1 store-operated calcium channel. Proc. Natl Acad. Sci. USA 107, 4896–4901 (2010).

38. Wang, X. et al. Distinct Orai-coupling domains in STIM1 and STIM2 define the Orai-activating site. Nat. Commun. 5, 3183 (2014).

39. Zhang, S. L. et al. Genome-wide RNAi screen of Ca\textsuperscript{2+} influx identifies genes that regulate Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channel activity. Proc. Natl Acad. Sci. USA 103, 9357–9362 (2006).

40. Zal, T. & Gascoigne, N. R. Photobleaching-corrected FRET efficiency imaging of live cells. Biophys. J. 86, 3923–3939 (2004).