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Junctophilin-4, a component of the endoplasmic reticulum–plasma membrane junctions, regulates Ca^{2+} dynamics in T cells

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Orai1 and stromal interaction molecule 1 (STIM1) mediate store-operated Ca^{2+} entry (SOCE) in immune cells. STIM1, an endoplasmic reticulum (ER) Ca^{2+} sensor, detects store depletion and interacts with plasma membrane (PM)-resident Orai1 channels at the ER–PM junctions. However, the molecular composition of these junctions in T cells remains poorly understood. Here, we show that junctophilin-4 (JP4), a member of junctional proteins in excitable cells, is expressed in T cells and localized at the ER–PM junctions to regulate Ca^{2+} signaling. Silencing or genetic manipulation of JP4 decreased ER Ca^{2+} content and SOCE in T cells, impaired activation of the nuclear factor of activated T cells (NFAT) and extracellular signaling-related kinase (ERK) signaling pathways, and diminished expression of activation markers and cytokines. Mechanistically, JP4 directly interacted with STIM1 via its cytoplasmic domain and facilitated its recruitment into the junctions. Accordingly, expression of this cytoplasmic fragment of JP4 inhibited SOCE. Furthermore, JP4 also formed a complex with junctate, a Ca^{2+}-sensing ER-resident protein, previously shown to mediate STIM1 recruitment into the junctions. We propose that the junctate–JP4 complex located at the junctions cooperatively interacts with STIM1 to maintain ER Ca^{2+} homeostasis and mediate SOCE in T cells.

junctophilins | ER–PM junctions | store-operated Ca^{2+} entry | STIM1 | Orai1

The endoplasmic reticulum (ER)–plasma membrane (PM) junctions are ubiquitous structures essential for intermembrane communications (1–3). These junctions play an important role in lipid transfer and regulation of Ca^{2+} dynamics, including ER Ca^{2+} homeostasis and Ca^{2+} entry after receptor stimulation (1, 4). Four major categories of components of the ER–PM junctions have been identified so far: (*i*) dyad/triad junctional proteins in the heart and skeletal muscle (e.g., junctophilins and junctin), (*ii*) ER-resident vesicle-associated membrane protein-associated proteins (VAPs) that form the lipid transfer machinery by interacting with phospholipid-binding proteins, (*iii*) extended synaptogamin-like proteins (E-Syts) that tether membranes, and (*iv*) the Orai1–stromal interaction molecule 1 (STIM1) complex that forms the primary Ca^{2+} channel in T cells, the Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Among these proteins, the dyad/triad junctional proteins and the Orai1–STIM1 complex are known to play a crucial role in Ca^{2+} dynamics, including excitation–contraction coupling in muscle and store-operated Ca^{2+} entry (SOCE) in immune cells, respectively (2, 5).

Stimulation of T-cell receptors (TCRs) triggers activation of SOCE primarily mediated by the PM-resident Orai1 channels and ER-resident STIM1 protein that senses ER Ca^{2+} concentration (6–11). Upon store depletion, STIM1 translocates and interacts with Orai1 at the preformed ER–PM junctions (12, 13). STIM1 uses two major mechanisms to translocate into the ER–PM junctions: by interactions with phosphatidylinositol-4,5-bisphosphate (PIP_2) in the PM via its C-terminal polybasic residues and by interaction with Orai1 or the ER-resident junctate proteins (14, 15). Recently, septin filaments were shown to play a role in PIP_2 enrichment at the ER–PM junctions before STIM1 recruitment (16). Subsequently, membrane-tethering VAP and E-Syt proteins were shown to be important for PIP_2 replenishment after store depletion (17). The importance of protein interaction in STIM1 recruitment was demonstrated by a STIM1ΔK mutant truncated in its C-terminal polybasic domain. Interaction with Orai1 or junctate facilitated recruitment of this PIP_2 binding-deficient mutant into the junctions (15, 18, 19). It was thought that the roles of dyad/triad junctional proteins are limited to muscle cells. However, identification of junctate as a STIM1-interacting partner implied that some components (or homologs) of ER–PM junctions in excitable cells may be shared in immune cells.

The junctophilin family consists of four genes (JP1, JP2, JP3, and JP4) that are expressed in a tissue-specific manner and are known to form ER–PM junctions in excitable cells (20, 21). Junctophilins contain eight repeats of the membrane occupation and recognition nexus (MORN) motifs that bind to phospholipids in the N terminus and a C-terminal ER membrane-spanning transmembrane segment (20, 22). In this study, we observed expression of JP4 in both human and mouse T cells, which was further enhanced by TCR stimulation. Depletion or deficiency of JP4 reduced ER Ca^{2+} content, SOCE, and activation of the nuclear factor of activated T cells (NFAT) and ERK mitogen-activated protein kinase (MAPK) pathways. Mechanistically, JP4 depletion reduced accumulation of STIM1 at the junctions without affecting the number and length of the ER–PM junctions. We observed a direct interaction between the cytoplasmic regions of JP4 and STIM1, and, correspondingly, overexpression of the STIM1-interacting JP4 fragment had a dominant negative effect on SOCE. Finally, we identified a protein complex consisting of JP4 and junctate at the ER–PM junctions, which may have a synergistic

Significance

Distinct membranes separate cellular organelles, and communication between organelles occurs primarily at the interorganelle membrane junctions, which are established by junctional proteins. The junctions between the endoplasmic reticulum (ER) and the plasma membrane (PM) are essential for lipid transfer and Ca^{2+} dynamics; however, little is known about the composition of these junctions in T cells. We identified a protein complex containing junctophilin-4 and junctate as components of the ER–PM junctions that regulate Ca^{2+} dynamics in T cells by interacting with stromal interaction molecule 1 (STIM1), an essential activator of store-operated Ca^{2+} channels. This study highlights an important role of junctional proteins in T cells and helps in uncovering the pathological mechanisms underlying human diseases due to mutations in these proteins.

Author contributions: J.S.W., S.S., and Y.G. designed research; J.S.W. and S.S. performed research; J.S.W., S.S., M.N., P.P., H.T., and Y.G. contributed new reagents/analytic tools; J.S.W., S.S., and Y.G. analyzed data; and S.S. and Y.G. wrote the paper.

The authors declare no conflict of interest.

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effect in recruiting STIM1 to the junctions. Therefore, our studies identify a PIP_2-independent, but protein interaction-mediated, mechanism by which the junctate–JP4 complex recruits STIM1 into the ER–PM junctions to maintain ER Ca^{2+} homeostasis and activate SOCE in T cells.

Results

JP4 Plays an Important Role in ER Ca^{2+} Homeostasis and SOCE in T Cells. To identify components of the ER–PM junctions in T cells, we examined transcript expression of various molecules, including junctional proteins in excitable cells (calumin, MG29, and JP1 to -4), proteins involved in lipid modification and transfer (VAP-A, -B, and -C and TMEM16A and -B), and membrane-tethering proteins (E-Syt1, E-Syt2, and E-Syt3) in resting and stimulated Jurkat cells, a leukemic T-cell line. Among these candidates, mRNA expression of JP4 was induced by stimulation in both Jurkat and murine primary T cells (Fig. 1*A* and Fig. S1*A*). Increased mRNA expression of JP3 was also observed, but only in Jurkat cells. Consistent with these mRNA analyses, expression of JP4 protein in Jurkat cells was induced after stimulation (Fig. S1*B*). JP4 is the least understood member of the junctophilin family and, together with JP3, is known to play an important role in neurons. Although JP1 and JP2 have specific roles in skeletal and cardiac muscle cells, respectively, JP3 and JP4 have redundant function in the brain. *Jph3* or *Jph4* single knockout mice do not show obvious abnormalities; however, deletion of both genes causes severe growth retardation and premature death in mice, possibly due to impaired neuronal function (23). However, so far, the role of JP4 in other cell types has not been examined.

To determine the role of JP4 in T cells, we depleted its expression in Jurkat cells using short hairpin RNAs (shRNA) (selected from five different shRNAs) (Table S1). This hairpin reduced JP4 transcript and protein expression by ∼60% (Fig. 1*B*). JP4-depleted Jurkat cells showed reduced SOCE after passive depletion of the ER Ca^{2+} stores with thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump or active depletion using anti-CD3 antibody cross-linking (Fig. 1*C*). Interestingly, ER Ca^{2+} content was also reduced in thapsigargin-treated JP4-depleted cells. We confirmed this observation using a strong ionophore, ionomycin, to ensure complete depletion of the ER Ca^{2+} stores (Fig. 1*D*). The specific role of JP4 in ER Ca^{2+} content and SOCE reduction was validated by rescue of these phenotypes in JP4-depleted cells expressing siRNA-resistant cDNA (Fig. 1*E*). Together, these results suggested an important role of JP4 in ER Ca^{2+} homeostasis and SOCE in T cells.

JP4 Deficiency Affects ER Ca^{2+} Homeostasis, SOCE, and Cytokine Production in Primary T Cells. Next, we examined the physiological role of JP4 in T cells isolated from JP4 knockout mice (23). As expected, JP4 expression was abrogated in JP4 knockout primary naive $CD4^+$ T cells (Fig. 2*A*). Furthermore, we observed a significant reduction in SOCE in JP4-deficent naive T cells after passive store depletion or TCR cross-linking (Fig. 2 *B* and *C*). Consistent with Jurkat cells, JP4 knockout primary T cells also showed a significant reduction in ER Ca^{2+} content (Fig. 2*B*). In addition, JP4-deficient effector T cells differentiated under nonpolarizing conditions also showed diminished SOCE after TCR cross-linking (Fig. 2*D*). These results confirm an important role of JP4 in ER Ca^{2+} homeostasis and SOCE.

To investigate physiological outcomes of reduced SOCE in JP4-depleted cells, we examined Ca^{2+}-dependent cytokine production. Accordingly, we observed reduced IL-2 expression in JP4-depleted cells (Fig. S2*A*). These results were supported by reduced NFAT-dependent luciferase expression in JP4-depleted cells. Furthermore, JP4-depleted cells also showed reduced expression of CD69, a T-cell activation marker, which is less dependent on the Ca^{2+}-NFAT pathway (Fig. S2*B*). These results prompted us to examine the role of JP4 in activation of other signaling pathways downstream of TCR activation, including those of MAPKs—ERK, p38, and c-Jun N-terminal kinase (JNK). Although phosphorylation of p38 and JNK remained unaffected, that of ERK

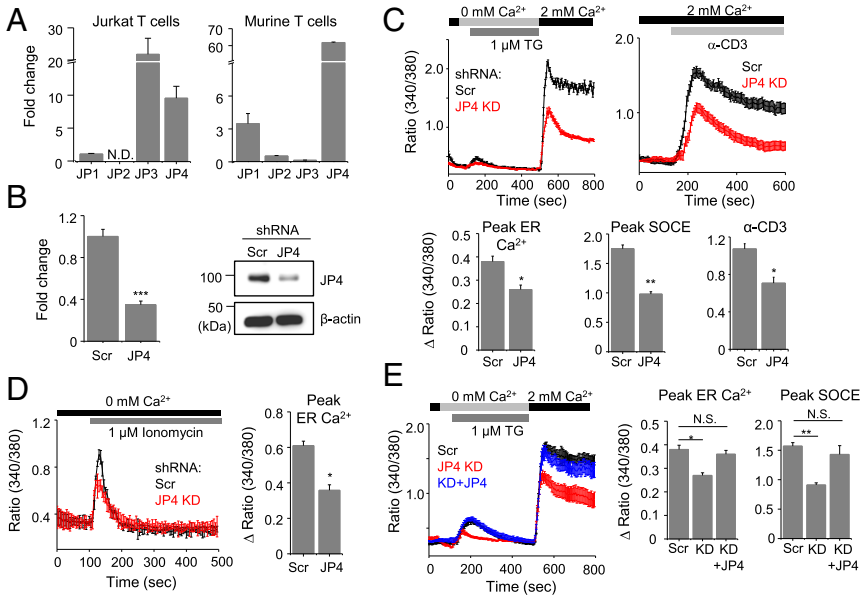

Fig. 1. Decreased ER Ca^{2+} content and SOCE in JP4-depleted Jurkat cells. (*A*) Expression of junctophilin transcripts in Jurkat (*Left*) or murine primary $CD4^+$ (*Right*) T cells with or without stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (6 h). Fold change depicts normalized mRNA levels relative to those under resting conditions. (*B*) Transcript (*Left*) and protein (*Right*) level of JP4 in Jurkat cells expressing control (Scr) or JP4-depleting shRNA (JP4). The transcript data show mean ± SEM of triplicates. (*C*) SOCE measurement in Jurkat cells expressing control (Scr) and JP4-depleting (JP4 KD) shRNA. Intracellular stores were passively depleted with thapsigargin (TG) (1 μM) in Ca^{2+}-free solution, and SOCE was measured by perfusion with 2 mM Ca^{2+}-containing solution (*Left*). TCR stimulation-induced SOCE was measured by cross-linking TCRs with α-CD3 antibody in the presence of external solution containing 2 mM Ca^{2+} (*Right*). (*D*) Measurement of ER Ca^{2+} content in Jurkat cells expressing control (Scr) and JP4-depleting shRNA after treatment with ionomycin (iono) (1 μM) in Ca^{2+}-free solution. (*E*) Measurement of SOCE in Jurkat cells expressing control (Scr), JP4-depleting (JP4 KD) shRNA, or JP4-depleting shRNA plus siRNA-resistant JP4 after passive store depletion as described in *C*. In *C–E*, traces show averaged (±SEM) responses from 30 to 50 cells, and bar graph shows averaged peak $[Ca^{2+}]_i$ ± SEM from three independent experiments. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

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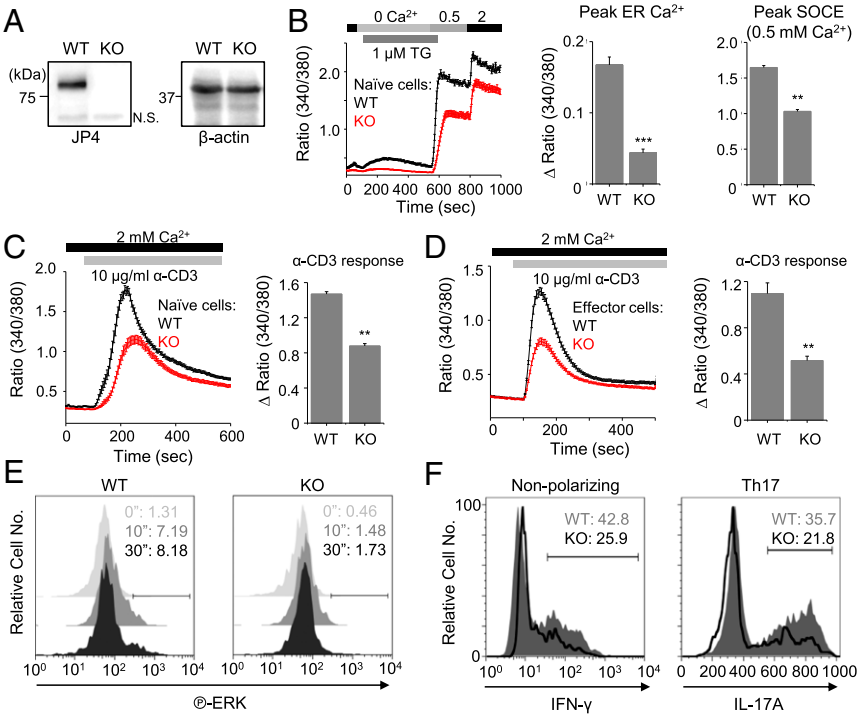

Fig. 2. Decreased Ca^{2+} signaling and effector function in JP4-deficient primary $CD4^+$ T cells. (*A*) Immunoblot for detection of JP4 (*Left*) or β-actin (*Right*) in lysates from WT and JP4-deficient naive $CD4^+$ T cells. N.S., nonspecific band. (*B*) SOCE measurement in WT and JP4-deficient (KO) naive T cells after passive store depletion with thapsigargin (TG) (1 μM) in Ca^{2+}-free solution and addition of 0.5 and 2 mM Ca^{2+}-containing solution. (*C*) SOCE measurements from WT and KO naive $CD4^+$ T cells after TCR cross-linking with α-CD3 antibody in 2 mM Ca^{2+}-containing solution. (*D*) SOCE measurement from WT and KO effector $CD4^+$ T cells cultured under nonpolarizing conditions after TCR cross-linking as described in *SI Materials and Methods*. In *B*–*D*, traces show averaged (± SEM) responses from 30 to 50 cells, and bar graph shows averaged peak $[Ca^{2+}]_i$ ± SEM from three independent experiments. (*E*) Representative flow plots showing phospho-ERK levels in WT and JP4-deficient naive T cells after stimulation with α-CD3 antibody (10 μg/mL) for indicated times. (*F*) Representative flow plots showing expression of IFN-γ and IL-17A in WT and JP4 KO T cells differentiated in the absence (*Left*) or presence (*Right*) of Th17-polarizing conditions for 4 d and restimulated with PMA and ionomycin (6 h). $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

was substantially diminished in JP4-depleted Jurkat cells (Fig. S2*C*). An important role of Ca^{2+} in activation of the RasGRP1-ERK pathway in T cells has already been established (24). Therefore, it is likely that reduced ERK activation in JP4-depleted cells is caused by decreased SOCE. Consistent with these results, JP4-deficient T cells also showed decreased ERK activation after TCR stimulation (Fig. 2*E*). Finally, JP4-deficient effector T cells, differentiated under nonpolarizing or T_H17-polarizing conditions, showed reduced expression of the effector cytokines IFN-γ and IL-17A, respectively (Fig. 2*F*). Together, these results suggest that JP4 is an important component of the Ca^{2+}-dependent NFAT and ERK pathways to mediate T-cell activation and effector T-cell function.

JP4 Controls ER Ca^{2+} Homeostasis and SOCE by Regulating STIM1 Function. To elucidate the molecular mechanism of JP4 in regulation of ER Ca^{2+} content and SOCE, we first examined its localization in T cells. Confocal analyses showed colocalization of JP4 with PM markers as well as an ER marker in the peripheral ER close to the PM, validating its localization to the ER–PM junctions (Fig. S3). Furthermore, expression of Orai1 or STIM1 was not altered in JP4-depleted cells (Fig. S4*A*), suggesting that reduced SOCE is not due to altered expression of the CRAC channel components. Recently, it was shown that, in addition to mediating SOCE, STIM1 is also important for the replenishment of ER Ca^{2+} stores in resting Purkinje neurons (25). Similarly, in STIM1-depleted T cells, we observed reduction in both the ER Ca^{2+} content and SOCE (Fig. S4 *B* and *C*). Importantly, expression of STIM1 in JP4-depleted Jurkat cells significantly rescued the decreased ER Ca^{2+} content and SOCE, indicating that JP4 may regulate STIM1 function (Fig. 3*A*).

To understand how JP4 regulates STIM1 function, we examined their localization under resting and store-depleted conditions in HEK293 and Jurkat cells. In HEK293 cells, under resting conditions, mCherry-JP4 localized to the PM-proximal areas whereas STIM1-YFP was primarily in the ER (Fig. S5*A*). After store depletion, STIM1 was primarily detected in the periphery at the ER–PM junctions with JP4. To monitor the kinetics of accumulation of STIM1 together with JP4 at the ER–PM junctions, we used live-cell total internal reflection fluorescence (TIRF) microscopy. Under resting conditions, JP4 showed a punctated localization at the junctions, and, after store depletion, STIM1 was recruited to those JP4-marked junctions (Fig. S5*B*). Notably, the fluorescence intensity of JP4 did not significantly change between resting and store-depleted conditions. Truncation of either the phospholipid-binding MORN motifs (ΔMORN) or the ER membrane traversing TM segment (ΔTM) abolished JP4 colocalization with STIM1 at the junctions, suggesting their importance in STIM1 recruitment (Fig. S5*C*). Furthermore, we observed an influence of the site of attachment of the fluorescent protein on JP4 and STIM1 colocalization. The presence of mCherry in the N terminus of JP4 did not influence its colocalization with STIM1 whereas that in the C terminus (ER-luminal side) inhibited colocalization. These results suggested that mCherry fusion to the ER-luminal side of JP4 inhibits STIM1 from accessing the JP4-containing ER–PM junctions, possibly due to space constraints. Therefore, we used only the mCherry-JP4 construct for subsequent studies.

Next, we examined the localization of JP4 with STIM1 in T cells. Similar to HEK293 cells, TIRF microscopy showed enhanced colocalization of JP4 and STIM1 after passive store depletion in Jurkat cells (Fig. 3*B*). To examine their accumulation upon physiological stimulation, we monitored their localization at the contact site between T cells and anti-CD3 antibody-coated coverslips. TCR engagement induces accumulation and colocalization of Orai1 and STIM1 in clusters at the site of stimulation (26, 27). Upon T-cell receptor stimulation, we observed specific localization of JP4 near the center of the cell whereas ZAP70, a protein tyrosine kinase involved in proximal TCR signaling, accumulated in clusters throughout the footprint of the cell (Fig. 3*C*). Interestingly, we observed a strong colocalization between JP4 and STIM1 at the center of the contact sites. Together with previous observation that STIM1 colocalizes with Orai1 upon TCR stimulation (27), these data show the presence of JP4 at the site of Orai1 and STIM1 accumulation. Finally, to understand how JP4 affected STIM1 recruitment into the junctions, we examined localization of endogenous STIM1 in control or JP4-depleted Jurkat T cells. In control cells, endogenous STIM1 showed prominent accumulation at the PM proximal regions after store depletion (Fig. 3*D*, white arrows); however, the area, number, and size of STIM1-containing clusters was significantly reduced in JP4-depleted Jurkat cells. Together, these results suggested that JP4 is localized to the ER–PM junctions in resting cells and plays an essential role in STIM1 recruitment into these junctions after store depletion.

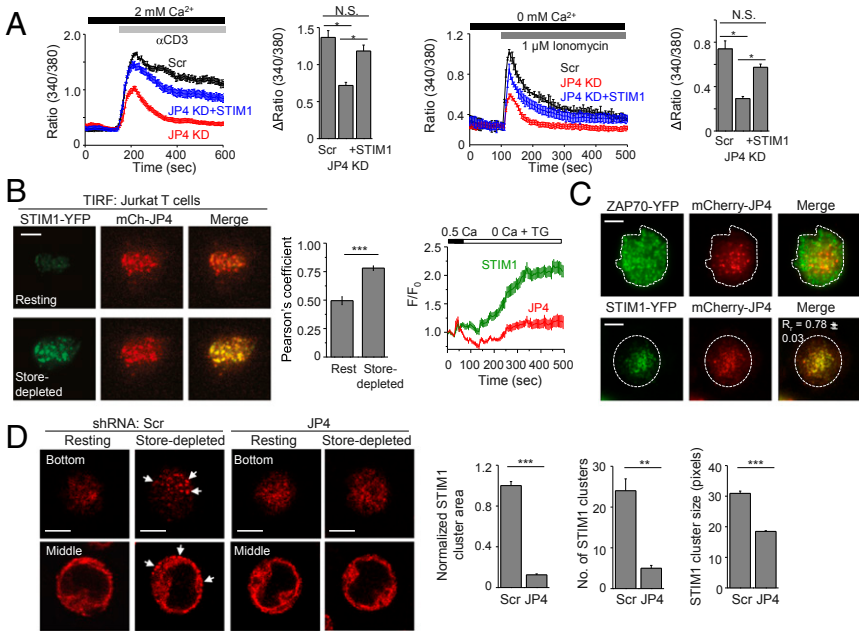

Fig. 3. JP4 is important for STIM1 recruitment at the ER–PM junctions. (*A*) Rescue of decreased SOCE and ER Ca^{2+} content in JP4-depleted Jurkat cells by STIM1 expression. Measurement of SOCE after TCR cross-linking in control (Scr), JP4-depleted (JP4 KD) cells, and JP4 KD cells expressing STIM1 (JP4 KD + STIM1) in the presence of external solution containing 2 mM Ca^{2+} (*Left*). These cells were also treated with ionomycin (1 μM) in Ca^{2+}-free solution to measure the ER Ca^{2+} content (*Right*). Traces show averaged (±SEM) responses from 30 to 50 cells, and bar graph shows averaged peak $[Ca^{2+}]_i$ ± SEM from three independent experiments. (*B*) Representative TIRF images of a Jurkat cell expressing STIM1-YFP and mCherry-JP4 under resting conditions (*Top*) or after store depletion with TG (*Bottom*). Bar graph (*Right*) shows average Pearson's correlation coefficient values (±SEM) from 11 cells. Line graph shows the time course of change in normalized fluorescence intensity (average ± SEM) from 11 cells. (*C*) Representative TIRF images of Jurkat cells expressing ZAP70-YFP (*Top*) or STIM1-YFP (*Bottom*) with mCherry-JP4 (*Top*) after TCR stimulation on α-CD3 antibody-coated coverslips. The merge panel shows average Pearson's correlation coefficient values (±SEM) from 8 cells. (*D*) Representative confocal images showing localization of endogenous STIM1 in Scr (*Left*) and JP4-depleted (*Right*) Jurkat cells before or after store depletion with TG. Two different sections from the center ("Middle") or the footprint ("Bottom") of the cell are shown. Arrows point toward STIM1 clusters. Bar graphs show normalized area (*Left*), number (*Center*), and size (*Right*) of STIM1-containing clusters from 8 to 12 cells after store depletion. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. (Scale bars: 5 μm.)

JP4 Physically Interacts with STIM1 to Regulate SOCE. We envisaged two possible mechanisms via which JP4 can regulate STIM1 translocation, and thus SOCE. These mechanisms include affecting the formation of the ER–PM junctions in T cells as a structural component or recruiting STIM1 into the junctions via protein interaction. To differentiate between these possibilities, we examined the numbers and area of the ER–PM junctions in control or JP4-depleted cells by TIRF and electron microscopy. However, we did not observe any reduction in the area, number, or length of the PM-proximal ER tubules in JP4-depleted Jurkat cells (Fig. 4*A* and Fig. S6). These results suggest that JP4 is not a crucial structural component for tethering of the PM and ER membranes in T cells or that other junctional proteins may compensate in formation of the ER–PM junctions. In any case, our data show that a decrease in SOCE by JP4 depletion or deletion was not caused by reduced ER–PM junctions.

To examine whether JP4 recruits STIM1 to the junctions by physical interaction, we checked for their interaction using immunoprecipitation and pulldown analysis. In coimmunoprecipitation experiments, we could detect GFP-JP4 only in the immunoprecipitates of HEK293 cells expressing FLAG-STIM1, but not in those without STIM1 (Fig. 4*B*). Conversely, we detected STIM1 only in the immunoprecipitates from HEK293 cells expressing FLAG-JP4. In both the cases, we observed a substantial increase in their interaction after store depletion. To narrow down the interaction domain, we generated fragments of both JP4 and STIM1 for GST pulldown analyses. Because JP4 does not contain any ER-luminal domain, we checked its interaction with the cytoplasmic fragments of STIM1 corresponding to the coiled-coil domains (CC) 1 and 2 (amino acids 250–400), the Orai1-interacting domain containing CC2 and CC3 (amino acids 342–448), the serine and threonine-rich region (amino acids 400–600), and the C-terminal PIP_2-interacting domain (amino acids 600–685). GST pulldown analyses showed a direct interaction of JP4 with the STIM1 fragment containing both the CC1 and CC2 regions (Fig. 4*C* and Fig. S7*A*). Further truncations of either CC1 or CC2 abolished their interaction. These results suggested that both CC1 and CC2 regions of STIM1 are required for its interaction with JP4. We also identified a cytoplasmic fragment of JP4 lacking the PM-binding MORN domain and the TM segment (amino acids 328–606; ΔMORN/TM) to be sufficient for interaction with STIM1. To summarize, these studies identified the cytoplasmic regions of both JP4 and STIM1 necessary for their interaction.

High overexpression of JP4 induced STIM1 clustering at the junctions even without store depletion, most likely by protein interaction (Fig. S7*B*). However, such recruitment of STIM1 did not increase the resting cytoplasmic $[Ca^{2+}]$, unlike the STIM1 EF-hand mutant, which constitutively induces Ca^{2+} entry. These results suggest that JP4 can recruit STIM1 into the junctions but cannot induce its open conformation to activate Ca^{2+} entry. We surmised that trapping of STIM1 by JP4 should reduce the time required for its translocation into the junctions to induce SOCE. Accordingly, we observed an increased rate of activation of SOCE in Jurkat cells overexpressing JP4 after TCR cross-linking or passive store depletion in the presence of a 2-mM Ca^{2+}-containing external solution (Fig. S7 *C* and *D*). Conversely, we also observed a reduced rate of Ca^{2+} entry in JP4-depleted or deleted T cells under similar conditions (Fig. S7*E*). Together, these results suggest that JP4 is involved in efficient STIM1 recruitment into the junctions by capturing it after store depletion via direct interaction.

Our biochemical analyses identified binding of STIM1 to the ΔMORN/TM fragment of JP4. We hypothesized that overexpression of this cytoplasmic fragment should inhibit STIM1 accumulation and reduce SOCE. Accordingly, among various fragments, the ΔMORN/TM fragment of JP4 showed a strong inhibition of SOCE in Jurkat cells (Fig. 4*D*). None of these fragments could rescue SOCE in JP4-depleted Jurkat cells, consistent with their lack of colocalization with STIM1 (Figs. S5*C* and S8*A*). The dominant negative effect of the cytoplasmic ΔMORN/TM fragment was dependent on the STIM1 expression level because it did not affect SOCE in HEK293T cells transiently overexpressing Orai1 and STIM1 whereas it reduced SOCE in HeLa cells moderately expressing these two proteins via stable expression (Fig. S8 *B* and *C*). It is interesting that the cytoplasmic ΔMORN/TM fragment showed a greater inhibition of SOCE than other fragments, including the ER-localized ΔMORN fragment, which should have a higher probability to interact with STIM1. At the current stage, we do not understand the exact mechanism behind this inhibitory effect, but it is possible that, in addition to the MORN domain, truncation of the TM segment is necessary for full exposure of the STIM1-interacting

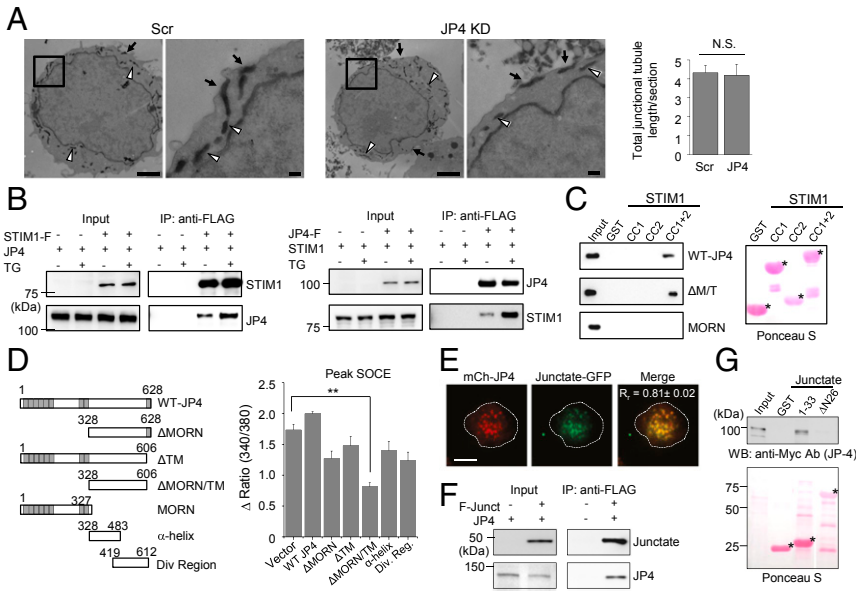

Fig. 4. JP4 interacts with STIM1 via the cytoplasmic domain and forms a protein complex with junctate. (*A*) Representative electron microscopy images of control and JP4-depleted Jurkat cells expressing HRP-ER and processed for HRP cytochemistry showing ER tubules at the ER–PM junctions (arrows) and in the cytoplasm (white arrowheads). Bar graph shows the total length of the junctional HRP-tubules per section (normalized to the PM circumference) in control (n = 15) and JP4-depleted (n = 19) cells. (Scale bars: 2 μm; *Inset*, 0.2 μm.) (Fig. S6). (*B*) JP4 coimmunoprecipitates with STIM1. Immunoprecipitates of anti-FLAG antibody from HEK293 cells expressing FLAG-STIM1 and GFP-JP4 were immunoblotted for detection of STIM1 (anti-FLAG antibody) and JP4 (anti-GFP antibody), respectively (*Left*). For reverse immunoprecipitation, immunoprecipitates from cells expressing FLAG-JP4 and STIM1 were immunoblotted for detection of JP4 (anti-FLAG antibody) and STIM1 (anti-STIM1 antibody) (*Right*). Cells were left untreated or treated with thapsigargin for 5 min to deplete the ER Ca^{2+} stores before lysis. (*C*) JP4 interacts with the CC1 and CC2 region of STIM1. (*Left*) GST pulldown analyses using indicated fragments of STIM1 (CC1, amino acids 234–340; CC2, amino acids 363–389, CC1+2, amino acids 250–400) with full-length JP4, ΔMORN/ΔTM (ΔM/T), and the MORN domains. (*Right*) Ponceau S staining of the same blot. (*D*) SOCE measurement from Jurkat cells expressing full-length JP4 or its fragments. Schematic of full-length JP4 and its fragments used in this study (*Left*). Gray boxes indicate the N-terminal MORN domains or the C-terminal TM segment. Bar graph shows averaged peak SOCE ± SEM from three independent experiments (*Right*). $^{**}P < 0.005$. (*E*) Colocalization of JP4 with junctate at the ER–PM junctions. Representative TIRF images of a Jurkat cell expressing junctate-GFP and mCherry-JP4. Pearson's correlation coefficient (R_r; ± SEM) was calculated from 13 cells. (*F*) JP4 coimmunoprecipitates with junctate. HEK293 cells stably expressing FLAG-Junctate were transfected with a plasmid encoding GFP-JP4. FLAG immunoprecipitates were immunoblotted for detection of junctate (anti-FLAG antibody) and JP4 (anti-GFP antibody). (*G*) JP4 directly interacts with junctate. GST pulldown analysis was performed between the N-terminal 33 amino acid fragment of junctate ("1-33") or junctate lacking the N-terminal 26 amino acids (ΔN26) with full-length JP4 purified from insect cells. (*Bottom*) Ponceau S staining of the same blot.

domain. Together, these data suggest that JP4-STIM1 interaction is important for SOCE and that expression of the cytoplasmic ΔMORN/TM fragment is likely to sequester endogenous STIM1 from binding to JP4, and thus inhibit SOCE.

JP4–Junctate Protein Complex at the ER–PM Junctions in T Cells. Earlier, we had identified junctate as a component of the ER–PM junctions in T cells (15). One caveat to defining junctate as a component of the ER–PM junctions is that, unlike JP4, it is distributed throughout the ER membrane, not just the PM-proximal region. A possible explanation lies in the very short N terminus of junctate, which lacks obvious phospholipid-binding motifs. However, it is possible that junctate interacts with PM-resident or specific junctional proteins to localize to the ER–PM junctions to mediate STIM1 recruitment. Interestingly, in Jurkat cells coexpressing JP4 and junctate, we observed a significant colocalization between these proteins at the junctions (Fig. 4*E*). We could also detect JP4 in junctate immunoprecipitates, suggesting an interaction between these two proteins (Fig. 4*F*). Because JP4 lacks a significant ER-luminal domain, we surmised that interaction between the two proteins should involve their N-terminal cytoplasmic regions. GST pulldown experiments showed a direct interaction of the short N-terminal cytoplasmic fragment of junctate with full-length JP4, which was abolished by truncation of this N-terminal fragment (Fig. 4*G*). In addition, we found a synergistic effect of coexpression of both JP4 and junctate on NFAT-driven luciferase expression in Jurkat cells (Fig. S9*A*). Together, these observations suggest the presence of a protein complex consisting of JP4 and junctate at the ER–PM junctions. Because both the proteins are prelocalized to the junctions and interact with STIM1, it is likely that the JP4–junctate protein complex can have a synergistic effect on STIM1 recruitment into the ER–PM junctions.

Discussion

The importance of junctional proteins is highly emphasized in excitable cells (3, 28). Dyad or triad junctions are the primary sites for Ca^{2+} dynamics in cardiac or skeletal muscle cells. Specialized proteins connecting the plasma and the ER membranes reside within these junctions (3, 28, 29). These junctional proteins include various single transmembrane segment-containing proteins, such as junctophilins, junctin, junctate, mitsugumins, and sarcalumenin. However, it was not known whether these proteins are expressed and perform similar functions in nonexcitable cells. We found that, among them, JP4 is located at the ER–PM junctions in T cells, where it plays an essential role in both ER Ca^{2+} refilling as well as SOCE, primarily mediated by its interaction with STIM1. A schematic showing the proposed mechanism of activation of SOCE by JP4 is illustrated in Fig. S9*B*. Junctate and JP4 form a protein complex at the ER–PM junctions through the interaction between their cytoplasmic N termini. Strategic formation of protein complexes between the PM and ER-resident proteins to shape the junctions is common, as exemplified by the protein complexes formed by the PM-resident oxysterol-binding protein-related protein (ORP) and the ER-resident VAP proteins for lipid transfer (1). An important aspect of this junctate–JP4 protein complex is the capacity of both proteins to interact with STIM1, which can be important for increasing the efficiency and determining the site of STIM1 recruitment. The interaction between STIM1 and junctate are mediated by their ER-luminal regions (15) whereas that between STIM1 and JP4 is mediated by their cytoplasmic regions. It is technically difficult to demonstrate an increased affinity of STIM1 for the JP4–junctate complex vs. the individual proteins because of the qualitative nature of GST pulldown and immunoprecipitation techniques used. However, it is highly likely that the junctate–JP4 complex can have a synergistic effect on recruiting STIM1 to the junctions compared with individual proteins, considering their interactions with distinct regions of STIM1.

Two mechanisms for STIM1 recruitment into the ER–PM junctions have been reported. One depends on PIP_2 enrichment at the junctions and the other is mediated by protein interactions. JP4 contains phospholipid-binding MORN motifs, which are highly conserved among the JP family. Interestingly, the MORN motifs of junctophilins have been shown to bind with high affinity to phosphatidylinositol (PI) monophosphates PI(3)P, PI(4)P, and PI(5)P and phosphatidyl serine (PS) whereas its affinity for PIP_2 was lower, suggesting that JP4 may not play a significant role in PIP_2 enrichment at the ER–PM junctions to mediate STIM1 recruitment (30). Instead, our study shows that

JP4 forms a complex with junctate at the ER–PM junctions and recruits STIM1 into these junctions by protein interaction. Although JP4 can recruit STIM1 into the junctions, it is not sufficient to induce the open conformation of STIM1, necessary to activate SOCE. In addition to SOCE, JP4 is important for ER Ca^{2+} homeostasis. It is possible that the junctions defined by localization of JP4, junctate, and STIM1 play an important role in ER Ca^{2+} refilling. In addition to STIM1, STIM2, which has a lower Ca^{2+} binding affinity, has been shown to play an important role in this process (31, 32). Further studies are required to examine whether the JP4–junctate complex is necessary for STIM2 recruitment to affect ER Ca^{2+} refilling in T cells.

Another possible role of JP4 can be its involvement in the ER stress pathways. ER stress activates the unfolded protein response (UPR) that contributes to regulation of various intracellular signaling pathways, including Ca^{2+} and lipid signaling (33). ER stress responses help in controlling tissue damage and repair. However, under pathological conditions, ER stress-induced inflammation exacerbates diabetes, obesity, atherosclerosis, and cancer. A common feature of ER stress is ER Ca^{2+} depletion that can be sensed by the STIM proteins (34). The physiological role of the junctional proteins in ER stress needs further investigation for a better understanding of the human pathologies underlying neurodegenerative disorders, Huntington's disease-like 2 (HDL2), arterial fibrillation, and cardiac hypertrophy caused by point mutations in junctate or junctophilins (35, 36). In summary, we uncovered an unexpected role of JP4 in ER Ca^{2+} homeostasis and Ca^{2+} signaling in T cells. Mechanistically, JP4 interacts with junctate to form a protein complex at the ER–PM junctions to recruit STIM1 by direct protein interaction. This study will help in understanding the composition of the ER–PM junctions in T cells and in uncovering their possible role in the pathology of human diseases caused by mutations in these proteins.

Materials and Methods

Plasmids and Cells. GST-tagged truncated STIM1 fragments have been previously described (37). The cDNA encoding murine junctophilin-4 (JP4, clone ID 6810400) was purchased from Open Biosystems (GE Dharmacon). FLAG-, mCherry-, or eGFP-fused full-length JP4 and its fragments were subcloned into pMSCV-CITE-eGFP-PGK-Puro, pN1-mCherry, pC1-mCherry, or pEGFPC1 vectors using primers shown in Table S1. Please see *SI Materials and Methods* for details.

Mice. All animals were maintained in pathogen-free barrier facilities and used in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of California, Los Angeles.

Single-Cell Ca^{2+} Imaging, Total Internal Reflection Fluorescence, and Confocal Microscopy. Single-cell Ca^{2+} imaging of T Cells loaded with 1 μM Fura 2-AM was performed as previously described (38). Please see *SI Materials and Methods* for details.

Immunoprecipitation and GST Pulldown Analyses. GFP-JP4–transfected HEK293T cells stably expressing FLAG-tagged STIM1 or JP4 were lysed and centrifuged at 100,000 × *g* for 1 h before preclearing. Lysates were immunoprecipitated with anti-FLAG antibody and protein A/G-Sepharose. Immunoprecipitates were washed and analyzed by immunoblotting. For pulldown analyses, GST-fused STIM1 fragments (37) and precleared lysates from HEK293 cells transfected with plasmids encoding FLAG-tagged JP4 were incubated in binding buffer. After multiple washes, bound proteins were analyzed by immunoblotting. Please see *SI Materials and Methods* for details.

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