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Molecular basis of the CRAC channel

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Abstract

Ca^{2+} release-activated Ca^{2+} (CRAC) channels, located in the plasma membrane, are opened upon release of Ca^{2+} from intracellular stores, permitting Ca^{2+} entry and sustained $[Ca^{2+}]_i$ signaling that replenishes the store in numerous cell types. This mechanism is particularly important in T lymphocytes of the immune system, providing the missing link in the signal transduction cascade that is initiated by T cell receptor engagement and leads to altered expression of genes that results ultimately in the production of cytokines and cell proliferation. In the past three years, RNA interference screens together with over-expression and site-directed mutagenesis have identified the triggering molecule (Stim) that links store depletion to CRAC channel-mediated Ca^{2+} influx and the pore subunit (Orai) of the CRAC channel that allows highly selective entry of Ca^{2+} ions into cells.

Keywords

CRAC channel; T lymphocyte; Store-operated calcium; Stim; Orai

1. Introduction

This review will cover the discovery of Stim and Orai and their functional roles in store-operated Ca^{2+} influx, emphasizing our own contributions. Up front, we recognize that several other groups have been engaged in a similar quest; many of their key contributions are represented in accompanying articles in this series. We introduce the topic by first considering background information on calcium signaling in lymphocytes, the electrical description of CRAC current, and its physiological role in lymphocytes. Then we discuss how a candidate-based RNA interference (RNAi) screen led to the discovery of *Drosophila Stim*. In further work, we identified two functions of *Stim* and the mammalian homologue STIM1 (collectively referred to here as Stim1): the sensor function that initially detects the reduction of Ca^{2+} content in the lumen of the endoplasmic reticulum (ER); and the messenger function provided by Stim1 translocation to the plasma membrane to activate the CRAC channel. We further discuss our genome-wide RNAi screen that identified *Drosophila Orai* and additional genes that are required for CRAC channel activity; the recipe for greatly amplified CRAC current by co-expression of Stim + Orai; and the identification of amino acids that regulate Ca^{2+} selectivity, clinching the identification of Orai as the pore-forming component of the CRAC channel.

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1.1. Lymphocytes and the early activation pathway of an immune response

T lymphocytes require direct contact with antigen-presenting cells (APC) such as dendritic cells to become activated. In parallel with work on the CRAC channel, the Cahalan lab group, in collaboration with Ian Parker at UCI, has used two-photon microscopy to image living T cells and dendritic cells interacting within intact mouse lymph nodes [1,2]. Under basal conditions without antigen, contacts between dendritic cells and T cells are initiated by virtue of robust T cell motility in combination with extension and retraction of dendritic cell dendrites, leading to a very high frequency of transient T cell/dendritic cell interactions [3]. Dendritic cells capture antigens in the periphery and bring them into the lymph node, serving as APC to initiate the immune response. When antigen is present, T cell–dendritic cell contact durations are prolonged as Ca^{2+} signaling is initiated; and after several hours, T cells are stably associated in clusters with individual dendritic cells [4]. Finally, the T cells let go and begin to divide about 24 h after initially encountering the antigen. Our story about the CRAC channels focuses on the T cell Ca^{2+} signal that is initiated by contact with APC and is crucial for initiation of an adaptive immune response.

1.2. Ion channels in human T cells

Ca^{2+} influx across the plasma membrane is primarily responsible for elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) following TCR engagement. But how does calcium enter the cell? Even earlier, nearly 25 years ago and shortly after the patch-clamp method was developed, researchers in the Cahalan lab initiated the study of ion channels in lymphocytes [5]. At that time, the importance of Ca^{2+} signaling to lymphocyte activation had already been proposed [6], and our initial expectation was that calcium channels might be detected electrically. At first, we investigated K^+ channels that regulate the membrane potential of T cells (and thereby indirectly modulate Ca^{2+} signaling), but no Ca^{2+} current was detected. By combining single-cell Ca^{2+} monitoring with patch recording, Richard Lewis in the Cahalan lab found a tiny Ca^{2+}-selective current with an inwardly rectifying I–V and a very positive reversal potential in Jurkat T cells [7]. The Ca^{2+} current, only a few picoamperes per cell, activated spontaneously during whole-cell recording with dialysis of a Ca^{2+} chelator. The same current could also be activated by T cell receptor stimulation during perforated-patch recording in a close temporal relationship with the upstroke of the cytosolic Ca^{2+} signal monitored simultaneously with a fluorescent Ca^{2+}-indicator. The current could be blocked by external application of Ni^{2+} or Cd^{2+}, agents that also blocked the mitogen-evoked Ca^{2+} signaling. During activation of the current, single-channel currents and even increased current noise were not evident, indicating a low single-channel conductance. At the time, a possible link of the Ca^{2+} current across the surface membrane to the internal stores was discussed, but the relation to store depletion and to models for “capacitative calcium entry” (CCE) [8] was unclear. The current was further studied by other groups, including Richard Lewis at Stanford and Reinhold Penner in Göttingen, and was shown to be activated by thapsigargin and by intracellular IP_3 [9–11], strengthening its identity as a store-operated Ca^{2+} channel. It was given the name CRAC, for Ca^{2+} release-activated Ca^{2+} current, by Penner.

Fig. 1 highlights the function of ion channels during an encounter between a T cell and an APC that has processed the antigens specific for the T cell receptor expressed on the T cell. Very few channels are open initially; in fact only a few of the voltage-gated Kv1.3 channels in the cell suffice to maintain the resting membrane potential of about −50 mV [12]. Contact with peptide-MHC on the APC initiates the proximal signaling cascade involving adapter molecules and tyrosine kinases, resulting in phospholipase C activation and the formation of the two messengers, IP_3 and DAG. IP_3 opens the IP_3 receptor-channels localized in the ER membrane, which in turn releases Ca^{2+} from the ER and begins to elevate cytosolic Ca^{2+}. Depletion of the ER Ca^{2+} stores indirectly leads to Ca^{2+} influx through CRAC channels, driving $[Ca^{2+}]_i$ into the micromolar range. As $[Ca^{2+}]_i$ rises, Ca^{2+}-activated K^+ channels are opened and K^+ leaves

the cell, causing the membrane potential to become more negative to maintain the driving force for Ca^{2+} influx. The relative contribution of voltage-gated and Ca^{2+}-activated K^+ channels to regulate membrane potential and counterbalance the influx of Ca^{2+} depends upon the T cell subset and their activation state [13]. Ca^{2+} binds to calmodulin and activates calcineurin, a phosphatase that dephosphorylates a transcription factor NFAT, which then translocates to the nucleus where it binds to promoter regions to turn on expression of numerous genes, including the key cytokine, Interleukin-2 [14,15]. The pharmacological agent thapsigargin bypasses the upstream events in T cell signaling by inhibiting the SERCA pump in the ER membrane, which leads to depletion of the ER Ca^{2+} store and activation of CRAC channels that trigger subsequent events including gene expression. Thapsigargin was thus employed as a very useful tool in our RNAi screening effort, described in Sections 2 and 3 below.

1.3. Ca^{2+} signaling and gene expression during T cell activation

By the mid-1990s, the calcium dependence of gene expression for T cell activation was determined quantitatively in single-cell Ca^{2+} assays combined with NFAT reporter gene monitoring [15,16]. To activate this key transcriptional regulatory pathway, at least 1–2 h of elevated $[Ca^{2+}]_i$ is required. The Ca^{2+} signal may be oscillatory or sustained, and it is thought that oscillations in $[Ca^{2+}]_i$ may provide a means to activate transcription of different genes selectively and without the requirement for continuous Ca^{2+} elevation, which could activate cell death pathways [17,18]. The content of the internal Ca^{2+} store is limited in T cells, and Ca^{2+} influx from outside of the cell is required to elevate Ca^{2+} to a level that is required for specific gene expression during T cell activation. CRAC channel activity and downstream Ca^{2+} effects via the calcineurin/NFAT pathway are thus critical for changes in gene expression elicited by TCR stimulation, as evidenced by altered gene expression in T cells that lack functional CRAC channels from patients with severe combined immune deficiency (SCID) disorder [14].

1.4. CRAC channels at the start of the millennium

As of 2003, then, the biophysical properties of CRAC current had been characterized, as summarized in Table 1. The immunological importance of the CRAC current to the NFAT pathway and to T cell activation was also clear. However, neither the channel itself nor the mechanism that links store depletion to CRAC channel opening were known. The major breakthroughs finally came in 2005 and 2006 when we and other groups used RNAi to identify genes that were required and site-directed mutagenesis to define the functional role of two proteins, Stim1 and Orai1, in turning on and physically embodying the CRAC channel, respectively.

2. RNAi candidate screen leads to identification of Stim, the Ca^{2+} sensor and messenger for CRAC channel activation

2.1. Choice of S2 cells for RNAi that led to the screening breakthrough

In the late summer of 2000, three of us (JR, KO and KS) joined a new company (now TorreyPines Therapeutics Inc.) and as part of an early project sought a novel way to identify the molecular components of store-operated Ca^{2+} entry (SOCE), especially the elusive CRAC channel. Previous work had suggested certain TRP channels might be the molecular counterparts of both store-operated Ca^{2+} channels and CRAC channels, but biophysical properties of TRP channels and CRAC channels did not match, and it seemed the field was nibbling around the edges because it lacked a more systematic and direct way to attack the problem. Then a colleague, Craig Montell, brought to our attention a newly developed technique for suppressing gene function called RNAi, which had shown utility in plants, worms, and flies. In these cases, introduction to cells of a double-stranded RNA (dsRNA)

mimicking a specific mRNA sequence causes sequence-specific degradation of the corresponding endogenous mRNA, leading to the suppression of gene expression.

The *Drosophila* S2 cell line attracted our attention because its utility for studying Ca^{2+} signaling mechanisms was already known [19–24]. S2 cells were an attractive target for performing an RNAi-based screen to identify genes that controls SOCE because most fundamental cellular processes are conserved from *Drosophila* to mammals and the *Drosophila* genome had just been published earlier in 2000 [25]. Of great importance in the interpretation of such a screen, the TorreyPines group teamed-up with the Cahalan group at UC Irvine to show that S2 cells possess a store-operated Ca^{2+} current. A detailed patch-clamp analysis showed that the store-operated Ca^{2+} current in S2 cells was remarkably similar in biophysical characteristics to the CRAC current in human lymphocytes (Table 1) [26], so the choice of S2 cells turned out to be a good one. Both currents are highly Ca^{2+} selective ($P_{Ca}/P_{Na} > 1000$) and can be activated by maneuvers that deplete the ER Ca^{2+} store: cell dialysis with a Ca^{2+} chelator (passive store depletion); internal application of IP_3; or external application of thapsigargin or ionomycin. Both exhibit the characteristic inwardly rectifying *I–V* shape with a reversal potential >50mV and an unusually low single-channel conductance in the femtosiemen range. In the absence of external divalent cations, both currents are carried by monovalent cations and sodium is more permeant than cesium. The monovalent Na^+ current declines (depotentiation) over tens of seconds in the continued absence of external divalents. The pharmacological properties are also quite similar: currents can be suppressed by lanthanides (La^{3+}, Gd^{3+}), SKF96365 or 2-APB (high μM range); while the application of 2-APB at lower concentration potentiates both currents. A more detailed comparison of the biophysical properties of CRAC current in S2 and T cells is presented in Table 1, along with characteristics of CRAC current induced by expression of Stim + Orai. These initial findings validated the use of S2 cells for identifying SOC/CRAC channel components for us and other investigators. That S2 cells possess a CRAC-like channel may not be surprising, as the S2 cell line was originally derived from a primary culture of late stage (20–24 h old) *Drosophila melanogaster* embryos and, based upon gene expression patterns and phagocytic activity, is thought to represent a haemocyte or macrophage-like cell [27,28].

2.2. How RNAi works in S2 cells

The *Drosophila* S2 cell culture system is ideally suited for moderate- to high-throughput assays of gene function using RNAi-mediated gene silencing. This is because RNAi in S2 cells is simple, efficient, and long-lived. S2 cells can be treated with long, ~500 base-pair dsRNAs, which are taken up easily from the medium by the cells [29]. An advantage of S2 cells is that they do not require transfection reagents for uptake of dsRNAs. The dsRNAs appear to enter the cells through receptor-mediated endocytosis [30]. Once inside the cells, dsRNAs are processed to 21–23 nucleotide fragments called small interfering RNAs (siRNAs) by the action of the enzyme Dicer. The use of long dsRNA probes increases the likelihood of producing siRNAs that efficiently bind to the RNA-inducing silencing complex (RISC), thereby reducing optimization steps necessary in designing siRNA probes for RNAi. Once bound to the RISC complex, siRNAs initiate degradation of the complementary mRNA sequences and subsequent suppression of protein translation. These considerations enhance the feasibility of conducting a high-throughput RNAi-based screen in S2 cells. Since the *Drosophila* genome is much smaller and was better examined at the time than the human genome, S2 cells provided the ideal model system to test the role of candidate genes in CRAC channel function by systematically suppressing functional expression through RNAi.

2.3. Choice of candidates by bioinformatics

At the time we initiated our first RNAi screen, genome-wide dsRNA libraries were not available, so we chose a bioinformatics approach to identify potential candidate genes. At the

beginning, we tested about 20 genes that were either known TRP family members or TRP-related sequences. When suppression of these genes failed to affect store-operated Ca^{2+} entry in S2 cells, we searched the *Drosophila* genome for all genes annotated as ion channels in the GadFly database (release 2). In addition, we identified genes having an InterPro channel-like motif (e.g., PROSITE, PRINTS, PRODOM) and genes that shared a Multiple Expectation maximum for Motif Elicitation (MEME) profile built from members of the TRP family. From this list, we excluded genes we had already tested, well-characterized voltage-regulated channels, non-calcium channels, and other genes that had functional data published in the literature (e.g., *cacophony*, *eag*, *Glu-RIIA*, *Glu-RIIB*, *para*, *Sh*) retaining ~80% of the candidates from the Gadfly-derived list and ~90% of the motif-derived list. Finally, we selected other genes containing Ca^{2+} binding motifs, genes annotated as plasma membrane ATPases, or genes with putative roles in SOC influx, arriving at a final list of 150 genes that were then targeted by RNAi individually and tested. As it turned out, *Stim* was included in the list not only because of its putative Ca^{2+} binding domain, but also because a transient bug in the genome annotation identified *Stim* as having a potassium ion-channel motif. This highlights the fact that at the time of our initial screen the annotation of the fly genome was still changing. In addition, novel classes of genes with undefined sequence motifs and architectures could not be discovered by a bioinformatics approach. Thus, another key component of the CRAC channel was only discovered later using unbiased genome-wide RNAi screens (i.e., *olf186-F*, the *Drosophila* homolog of human Orai1 or CRACM1) [31–33].

2.4. How the initial candidate screen worked

The initial screen (Fig. 2) utilized a FluoroSkan single-channel fluorimeter to measure baseline and SOCE signals. S2 cells were treated with the appropriate dsRNAs in T-75 flasks for 5 days, harvested and plated in 96-well plates, and then loaded with the Ca^{2+} sensitive dye fluo-4 in buffer containing calcium. Cells were washed in calcium-free buffer and fluorescence levels were then monitored by the FluoroSkan. Initial fluorescence was recorded for a brief period to estimate basal Ca^{2+} levels, followed by addition of either vehicle or thapsigargin to discharge intracellular Ca^{2+} stores and activate store-operated Ca^{2+} channels. After a short incubation, $CaCl_2$ was added to the cells and a single fluorescence reading was recorded three min after Ca^{2+} addition as an index of store-operated Ca^{2+} influx in each well. This single-point assay was possible because in our hands the Ca^{2+} entry signal became stable 2–4 min after Ca^{2+} addition, and it greatly facilitated the throughput of the RNAi screen. It was with this assay that we identified the activity of *Stim* [34] (first revealed in a patent application published in 2004 with priority to 2003 [35], and presented publicly at the Biophysical Society meeting in February 2005), a finding consistent with a subsequent, independent report by Liou et al. [36]. We later used this basic paradigm in a genome-wide RNAi screen to identify the role of *olf186-F* (Orai) [33].

2.5. Validation of Stim and STIM1 by single-cell Ca^{2+} imaging and patch-clamp

Our initial study validated the requirement for Stim by single-cell Ca^{2+} monitoring and by whole-cell recording in S2 cells pretreated with dsRNAs for Stim or control genes [34]. In Ca^{2+} imaging experiments, we used a thapsigargin and Ca^{2+} readdition protocol to assess SOCE in single cells. Following addition of thapsigargin in the absence of external Ca^{2+}, a small transient rise in cytosolic free Ca^{2+} represents the release transient due to leak from the store into the cytoplasm. Subsequent readdition of external Ca^{2+} produces a very large Ca^{2+} signal that is due to Ca^{2+} influx through CRAC channels that are activated as a result of thagpsigargin-induced Ca^{2+} store depletion. In cells pretreated with dsRNAs for Stim, resting Ca^{2+} was unaltered and store content was normal, but Ca^{2+} influx upon readdition of external Ca^{2+} was profoundly inhibited. Patch-clamp experiments confirmed that knockdown of Stim by RNAi very effectively suppressed the CRAC current. There are two mammalian homologs of *Drosophila* Stim, STIM1 and STIM2. Suppression of STIM1 by treatment of HEK293 cells

with STIM1-specific siRNAs inhibited thapsigargin-evoked Ca^{2+} entry and Ca^{2+} influx induced by muscarinic receptor stimulation, without altering resting Ca^{2+} levels, Ca^{2+} release transients, or membrane potentials [34]. In our hands, suppression of STIM2 had less of an effect on SOCE than did STIM1 knockdown. In Jurkat T cells, we again confirmed that suppression of STIM1 led to a profound reduction of the Ca^{2+} signal and CRAC current, this time using a short hairpin loop (shRNA) construct to reduce STIM1 expression selectively.

2.6. Dual functional role of Stim1: ER Ca^{2+} sensor and messenger to the plasma membrane

In the experiments described above, RNAi knockdown demonstrated a functional requirement for Stim1 expression in supporting CRAC channel activity. But how does Stim1 function, as a single span transmembrane protein that does not even remotely resemble any known ion channel? *Drosophila* Stim and its mammalian homologs, STIM1 and STIM2, are type 1 membrane proteins. The signal peptide sequence at the amino terminus and one predicted transmembrane segment indicated that the amino (N-) terminal portion of the protein faces the extracellular fluid or the lumen of the ER. The conserved N-terminal EF-hand motif suggested a possible role of Stim1 in Ca^{2+} signaling, and since Stim1 did not resemble any known ion channel we formulated the idea that it may serve as a Ca^{2+} sensor to trigger the process of CRAC channel activation. The approach we took was to overexpress the wild-type (WT) Stim1 protein or Stim1 mutant proteins in S2 and Jurkat T cells [37].

Our strategy for a series of point mutations was to disrupt Ca^{2+} binding to the EF-hand domain while preserving the overall protein structure, targeting different conserved residues that had been shown in other EF-hand proteins and by crystal structures to be essential for Ca^{2+} binding (Fig. 3). By making several different mutations on the same sites (D to N or D to A, and E to Q or E to A; Fig. 3 ovals) and corresponding mutations in different species (Stim and STIM1), we tried to avoid false-positive results. The mutants of Stim or STIM1 were expressed in S2 or Jurkat T cells, respectively, by transfection, and calcium signaling was compared in native cells and cells overexpressing either wild-type (WT) or mutant Stim/STIM1. Expression was confirmed by Western blot which showed similar expression levels of WT and mutants proteins. The S2 cells overexpressing the WT *Stim* had no phenotype, whereas expression of the EF-hand mutants produced an elevation of resting $[Ca^{2+}]_i$ that we showed was due to constitutive activation of CRAC channels, since it was reduced to control levels by three inhibitors of CRAC current: 2-aminoethyldiphenyl borate (2-APB), SKF96365, and Gd^{3+}. In Jurkat T cells, a modest enhancement of thapsigargin-evoked Ca^{2+} influx was produced by overexpressing the WT protein, and expression of the EF-hand mutant elevated cytosolic Ca^{2+} levels, as seen with S2 cells. This rise in Ca^{2+} was also completely blocked by CRAC channel inhibitors. Importantly, there was no change in ER store content or release kinetics, leading to the conclusion that expression of the EF-hand mutants bypassed the normal requirement for store depletion and activated CRAC channels even though the store was still replete with Ca^{2+}. These findings suggested that Stim1 serves as an ER Ca^{2+} sensor that can trigger subsequent events when the Ca^{2+} store is depleted. According to this idea, Stim1 changes its state to Ca^{2+}-unbound upon store depletion or by crippling the EF-hand and is the effector that initiates a sequence of events leading to activation of the CRAC channel in the plasma membrane.

In parallel with these functional studies, we determined the distribution of the overexpressed STIM1 proteins by immunofluorescence using polyclonal antibodies specific to STIM1 and found a very different pattern of expression for WT and EF-hand mutant STIM1 [37]. The overexpressed WT STIM1 proteins were primarily localized in an ER-like pattern with some expression also visible at the plasma membrane, whereas the EF-hand mutant STIM1 proteins were predominantly seen at the plasma membrane in aggregations that we termed hotspots. Importantly, similar hotspots, or puncta, of native STIM1 were observed in several cell types,

including Jurkat T cells, primary human T cells, RBL cells, and PC12 cells, using the same polyclonal antibodies shortly following store depletion. We used SERCA as a *bona fide* marker of the ER for comparison to the native STIM1 pattern of subcellular localization which changed following store depletion. In normal Ringer solution, there was extensive co-localization of both proteins. But when the store was depleted by thapsigargin, co-localization was reduced, because the STIM1 protein had migrated and was predominantly seen in hotspots on the cell surface. In addition, a time-course study showed that this redistribution of STIM1 to the plasma membrane was rapid, overlapping with the time-course of CRAC channel activation. After presenting these data at the annual Biophysical Society meeting (Feb. 2005), we decided to explore the subcellular localization of STIM1 with higher resolution by electron microscopy. The polyclonal antibodies were conjugated to quantum dots that are both highly fluorescent and visible as particles by electron microscopy. By this technique, native STIM1 in T cells was shown to redistribute to the plasma membrane following Ca^{2+} store depletion.

In summary, our results indicate that Stim1 has two functions: using the EF-hand to sense luminal ER Ca^{2+} content; and migrating to the plasma membrane when the EF-hand senses lowered Ca^{2+} in the ER. A similar conclusion was reached using an EF-hand YFP-tagged STIM1 mutant to activate SOCE and YFP-tagged STIM1 to track the migration of STIM1 to the cell surface following store depletion [36]. The Ca^{2+} sensor hypothesis was strengthened by a subsequent *in vitro* biochemical study using a core N-terminus STIM1 construct [38]. An apparent dissociation constant (K_d) of 200–600 μM was measured, consistent with the functional range of Ca^{2+} levels within the ER upon store depletion.

2.7. A role for surface STIM1?

The presence of native STIM1 protein in both the plasma membrane and the ER was originally reported by using two different polyclonal antibodies against either N-terminus or C-terminus of STIM1 [39], and was confirmed by a later study [40]. By surface biotinylation, we found an increase in the surface-accessible native STIM1 protein upon store depletion in Jurkat T cells [37] that was confirmed in human platelets [41]. Yet, three reports showed that N-terminal YFP-, GFP-, and HA-tagged STIM1 is not extracellularly accessible following store depletion [36,42,43]. Results with tagged STIM1 constructs that fail to externalize indicate that acute surface exposure of STIM1 following Ca^{2+} store depletion may not be required for activation of the CRAC channel. Whether fluorescent protein or even smaller HA tags perturb STIM1 localization remains an issue. A recent study showed that N-terminal tagging of STIM1 with a small hexahistidine motif, but not with CFP, revealed cell surface exposure of STIM1 following thapsigargin treatment [44]. Thus, the presence of the larger fluorescent protein tag perturbed the re-localization of STIM1 into the plasma membrane. Several possibilities remain at this time, including a role for surface STIM1, in addition to STIM1 puncta next to the plasma membrane, in CRAC channel function. STIM1 on the plasma membrane was also reported to be essential for the activity of arachidonic acid-regulated Ca^{2+}-selective (ARC) channels [45].

3. Genome-wide RNAi screen leads to the identification of olf186-F (Orai), the CRAC channel pore-forming subunit

3.1. Screening the fly genome with calcium signals

Because work on Stim1 made it clear that the actual CRAC channel had not yet been identified, we went back to RNAi screening in June 2005 and performed an unbiased and quantitative genome-wide screen at Harvard's *Drosophila* RNAi Screening Center (DRSC) to isolate additional molecules essential for SOC influx, with the primary goal of identifying the CRAC channel itself [33]. Fig. 4 illustrates some aspects of the screening protocol. At the DRSC, the entire *Drosophila* genome is represented in a set of 384-well plates, each well containing a

dsRNA (amplicon) targeting a particular gene. Similar to the previous candidate screen that identified Stim, fluorescence from the Ca^{2+}-sensitive dye fluo-4 was monitored systematically in each well at three time points, corresponding to "basal" (resting intracellular free Ca^{2+}); "CCE" (TG-dependent Ca^{2+} influx) measured 4 min after adding thapsigargin; and "F_{max}" (maximal fluorescence to normalize for cell number) after adding TritonX-100. The screen was finished in a four-week period and generated a large data set covering the entire genome [3 fluorescence measurement per well × 384 wells per plate × 63 plates × 2 duplicate experimental runs → >145,000 data points]. The overall high reproducibility was confirmed by a scatter plot with the two values derived from each amplicon.

3.2. Analyzing the screening data

Next, the screening results were processed. To assign statistical significance to the fluorescence values, *z*-scores for each well (calculated as the value of individual well minus the average, then divided by the standard deviation) were calculated. Candidate hits in four groups were selected if basal/F_{max} or CCE/basal values were reduced or increased by more than three standard deviations from the mean. First, we computed the basal/F_{max} for each well to provide a number for the normalized resting intracellular free Ca^{2+} level. Two groups (1 and 2) of candidates (hits) were defined by *z*-scores <−3 or >3, indicating abnormally low or high resting Ca^{2+}, respectively. RNAi of these genes significantly influenced calcium homeostasis under resting conditions. Then, the CCE/basal ratio for each well was calculated to provide an index for the relative thapsigargin-evoked Ca^{2+} influx. This yielded an average value of about 2.1, and another two groups (3 and 4) were defined with *z*-scores <−3 or >3, indicating abnormally low or high store-operated Ca^{2+} entry, respectively. Assuming that RNAi of genes (e.g. Stim) that control SOCE do not influence the resting Ca^{2+} levels, overlapping candidates in groups 1 and 4 were removed from group 4, and overlapping candidates in groups 2 and 3 were removed from group 3 to avoid false-positive outcomes resulting from altered resting Ca^{2+} levels. The value of F_{max} was used to filter out wells in which cells did not grow or failed to adhere, using a *z*-score of F_{max} <−2 as a cutoff (confirmed by visual observation). In addition, we filtered out wells having more than five off-targets (non-specific RNAi effect). We then focused on filtered hits in group 3 with 75 remaining candidates, which should include the long-sought CRAC channel pore gene. The list was further narrowed down to those candidates with predicted transmembrane segments and with mammalian homologs; both are essential conditions for the CRAC pore subunit. In the end, a final list of 11 transmembrane protein hits was generated [33]. Gratifyingly, Stim was one of the strongest hits with a CCE/basal value of 1.26.

3.3. Olf186-F (Orai) as a conserved membrane protein with three human homologs

Uniquely among the filtered transmembrane protein hits, olf186-F (now renamed Orai), with a CCE/basal value of 1.31 and four predicted transmembrane segments, had no known function. Although only one member is represented in fly (dOrai, Fig. 5) and worm genomes, a conserved three-member gene family is present in all sequenced mammalian genomes. These are now renamed Orai1, Orai2, and Orai3 on human chromosomes 12, 7, and 16, respectively. Sequences and other key features are shown in Fig. 5. There are four predicted transmembrane segments in each Orai homolog which are all highly conserved, especially the first two transmembrane segments and the loop region in between. These provided hints for our follow-up mutagenesis strategy (see below).

3.4. Validation of hits by single-cell Ca^{2+} imaging and patch-clamp

Several final candidates were validated at the single-cell level by both Ca^{2+} imaging and patch-clamp analysis for direct RNAi effects on CRAC activity [33]. As for olf186-F (Orai), the vast majority of cells pretreated with dsRNA targeting olf186-F did not exhibit Ca^{2+} influx upon

store depletion and had greatly reduced CRAC current densities, whereas both resting Ca^{2+} level and store content were the same as in control cells. We also validated the SERCA pump, which could have been a false-positive hit since the screen was based on thapsigargin. In single-cell Ca^{2+} assays and whole-cell recording from cells pretreated with dsRNA to knock down SERCA, there was a highly significant reduction in thapsigargin-evoked Ca^{2+} entry and in CRAC current densities recorded during passive store depletion. It remains to be determined whether this reduction resulted from abnormally low Ca^{2+} store content (assayed by ionomycin-evoked store release) or elevated $[Ca^{2+}]_i$ levels. Syntaxin 5 was also validated as a genuine hit. Thus, by RNAi we confirmed with Ca^{2+} imaging and patch-clamp recording that Stim, Orai, SERCA, and Syntaxin 5 are each required for normal CRAC channel function.

An RNAi screen based on NF-AT translocation as a functional measurement was performed in DRSC at the same time [31]. Combined with genetic mapping of SCID patients which was initiated in 1996, Orai was also identified as an essential component for store-operated Ca^{2+} influx and the downstream NF-AT relocation into the nucleus. Moreover, a recessive point mutation in human Orai1 on chromosome 12 was isolated from SCID patients [31]. This channelopathy further reinforces the functional significance of CRAC channels (and Orai1) for the immune system. A third RNAi screen initiated several months later also identified the same gene (which was renamed as CRACM1 for CRAC modulator 1), together with another candidate (*dpr*3) called CRACM2 in the fly genome [32]. Remarkably, all three screens identified olf186-F (Orai, CRACM1) as an essential transmembrane protein.

3.5. Overexpression of Orai and Stim: amplified CRAC current

RNAi can test whether a particular molecule may be required for normal function. But in a complex multicomponent system, several required elements may be present. Thus, we sought to investigate effects of overexpression. We cloned the cDNA of Orai from S2 cells and expressed it with or without co-transfected Stim. When Orai was expressed alone, CRAC current density increased three-fold, a highly significant increase and in contrast to the lack of effect when Stim was overexpressed by itself. Importantly, when both genes were transfected, a huge CRAC current was recorded, and the kinetics of activation were accelerated compared to CRAC current induced by overexpression of Orai alone. The amplified CRAC current exhibited all the biophysical characteristics of native CRAC current in S2 cells (Table 1). These coexpression results provided further evidence that Orai may form the CRAC channel pore [33].

3.6. Mutations in Orai that affect CRAC channel Ca^{2+} selectivity

The ability of ion channels to select among various ions depends on interactions between amino acid side chains, ions and water molecules. Therefore, we sought to identify particular amino acids that, when mutated, resulted in altered ion selectivity of CRAC current following expression of Orai + Stim. Ion selectivity is usually accomplished by loop regions between transmembrane segments. It has been shown that negatively charged amino acids are the key elements for the Ca^{2+}-selectivity filter of voltage-gated Ca^{2+} channels and TRPV6 [46,47]. The loop between transmembrane segments 1 and 2 of the fly Orai gene and mammalian Orai family members is the most conserved (Fig. 5). We focused on negatively charged residues and prepared a series of alanine substitutions. Mutation of the conservative glutamate to alanine at position 180 of Orai resulted in inhibition of the background S2 CRAC current, indicating a dominant-negative action. A conservative point mutation at this position to aspartate dramatically altered the ion selectivity properties of CRAC current. Whereas a large inward current developed during passive store depletion when WT Orai + Stim were co-transfected, an outward current developed when the point mutant E180D Orai was expressed together with Stim. This was because the CRAC channel *I–V* characteristic displayed pronounced outward rectification, rather than the normal inward rectification of native or amplified CRAC current.

Ion substitution experiments clearly revealed a change from Ca^{2+} selective permeation to monovalent cation currents carried by Na^+ or Cs^+. Furthermore, several other properties related to ion selectivity were affected. In divalent-free solution, the relative Na^+ to Cs^+ permeability, P_{Na}/P_{Cs}, was reduced. The increase in Cs^+ permeability is consistent with a slightly enlarged selectivity filter. Furthermore, the monovalent currents failed to depotentiate. The dramatic alterations of ion selectivity by a point mutation at position 180 of Orai indicate that Orai is the pore-forming subunit of the channel, responsible for Ca^{2+} selectivity. Interestingly, three other mutants near 180 had reduced sensitivity to the channel blocker Gd^{3+} possibly by reduced charge screening resulting in a reduced local concentration of the trivalent blocker. Taken together, these results lead to the conclusion that Orai is a *bona fide* CRAC channel pore subunit [48]. A corresponding point mutation in Orai1 at position 106 results in similar changes [49, 50], although with some differences in rectification between the corresponding Orai and Orai1 mutants.

3.7. Interaction with Stim

So far, the CRAC channel gating mechanism has not been elucidated. The molecular events between Stim1 translocation and CRAC activation remain undefined. Both STIM1 and Orai1 accumulate at junctional sites where the ER and plasma membrane are closely apposed following store depletion [43,51]. In S2 cells, we found a greatly enhanced interaction between Orai and Stim upon store depletion, assessed by co-immunoprecipitation (co-IP), reflecting a dynamic interaction model for CRAC channel activation [48]. One possibility is that Stim delivers information about the store (empty) to Orai by direct physical contact, which in turn opens the channels. However, amino acid sequences required for the interaction and a possible requirement for other scaffolding proteins need to be further tested. As *Drosophila* Stim and Orai are both required to reconstitute CRAC channel activity in HEK cells (unpublished data), it will be interesting to see whether these two proteins alone form a minimal functional unit of CRAC channels. On the other hand, the redistribution of both STIM1 and Orai1 hint that there may be a finely tuned recruitment mechanism to generate a polarized activated CRAC channel distribution for Ca^{2+} signaling in microdomains. An interaction between mammalian STIM1 and Orai1 was also reported via co-IP assay without TG-treatment [50]. The interaction may be indirect or of low affinity as another co-IP based study showed that Orai1 did not associate with STIM1 in several detergent and salt conditions tested [52]. Further work is needed to reconcile these data and to determine how store depletion affects the interaction.

4. Overview and prospects

RNAi screening [31–34,36] and subsequent functional studies have clarified the molecular requirements and activation mechanisms of the CRAC channel, a sort of holy grail in the field of Ca^{2+} signaling. In human T cells, STIM1 senses ER Ca^{2+} content using an EF-hand motif near the N-terminus and translocates to the plasma membrane when the Ca^{2+} store is depleted [36,37]. The Ca^{2+} channel itself is formed from Orai1 in T cells, since a naturally occurring mutation within the first transmembrane segment abrogates channel activity in SCID patients [31], and a point mutant of a conserved glutamate residue between transmembrane segments 1 and 2 in *Drosophila* Orai or human Orai1 alters the Ca^{2+} ion selectivity of the channel [48–50].

The next challenge is to understand the detailed molecular composition and organization of CRAC channels, as a unique Ca^{2+} channel unrelated to other channel families. The subunit organization needs to be defined, and further site-directed mutagenesis with functional assays will provide more information. Ultimately a crystal or NMR structure will be essential to complete this mission. High-throughput screening may provide useful CRAC-specific blockers. Furthermore, the functional role of Stim and Orai homologs will need to be defined. The three human Orai homologs exhibit different tissue expression patterns. The fact that

normal Orai2 and Orai3 genes cannot compensate the mutated Orai1 gene in SCID patients indicates that the three members may have different physiological roles [52]. Genetic targeting is sure to provide fresh insights, as will *in vivo* pharmacological targeting.

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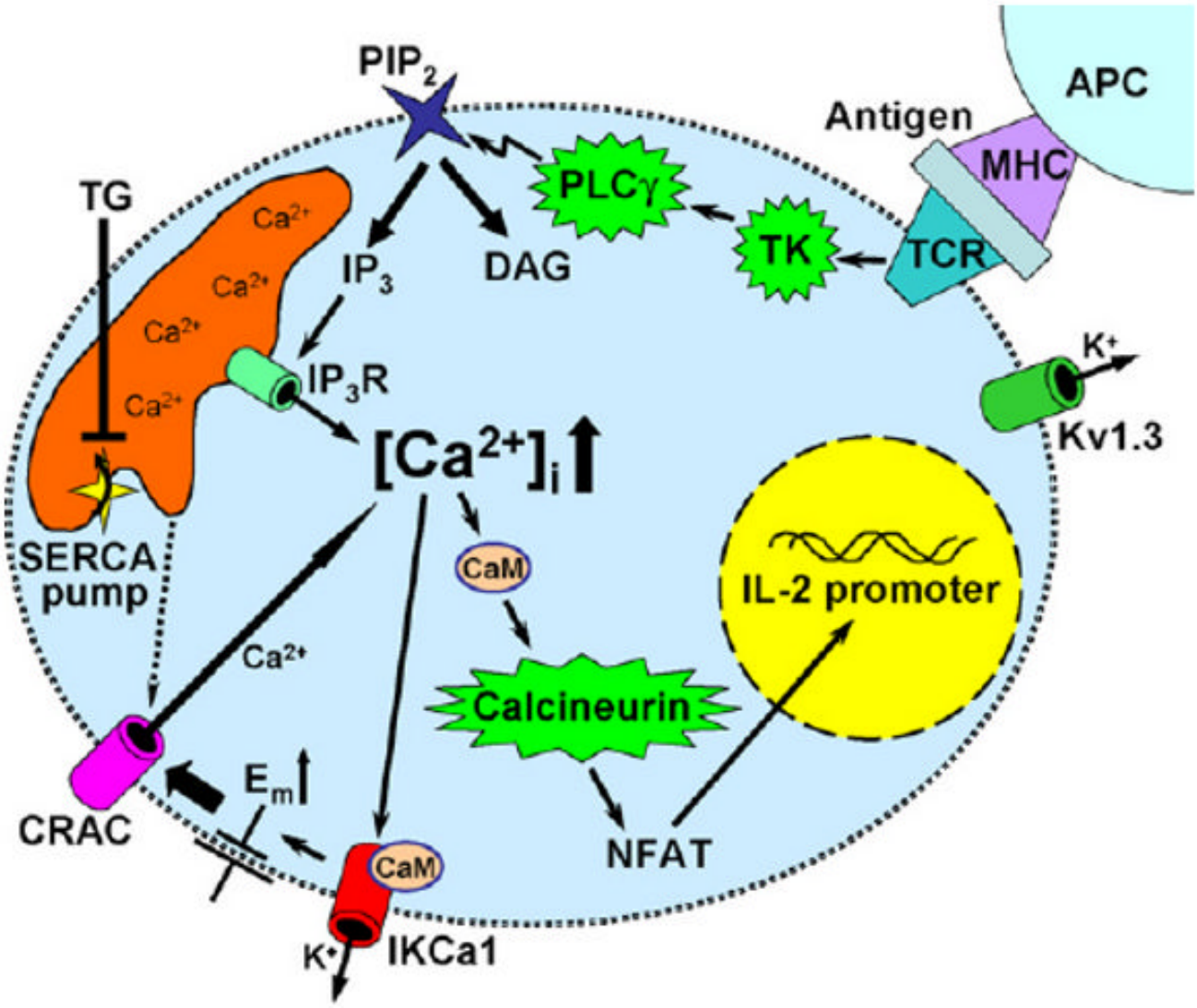

Fig. 1.
Ion channels and signaling pathways in T cells.

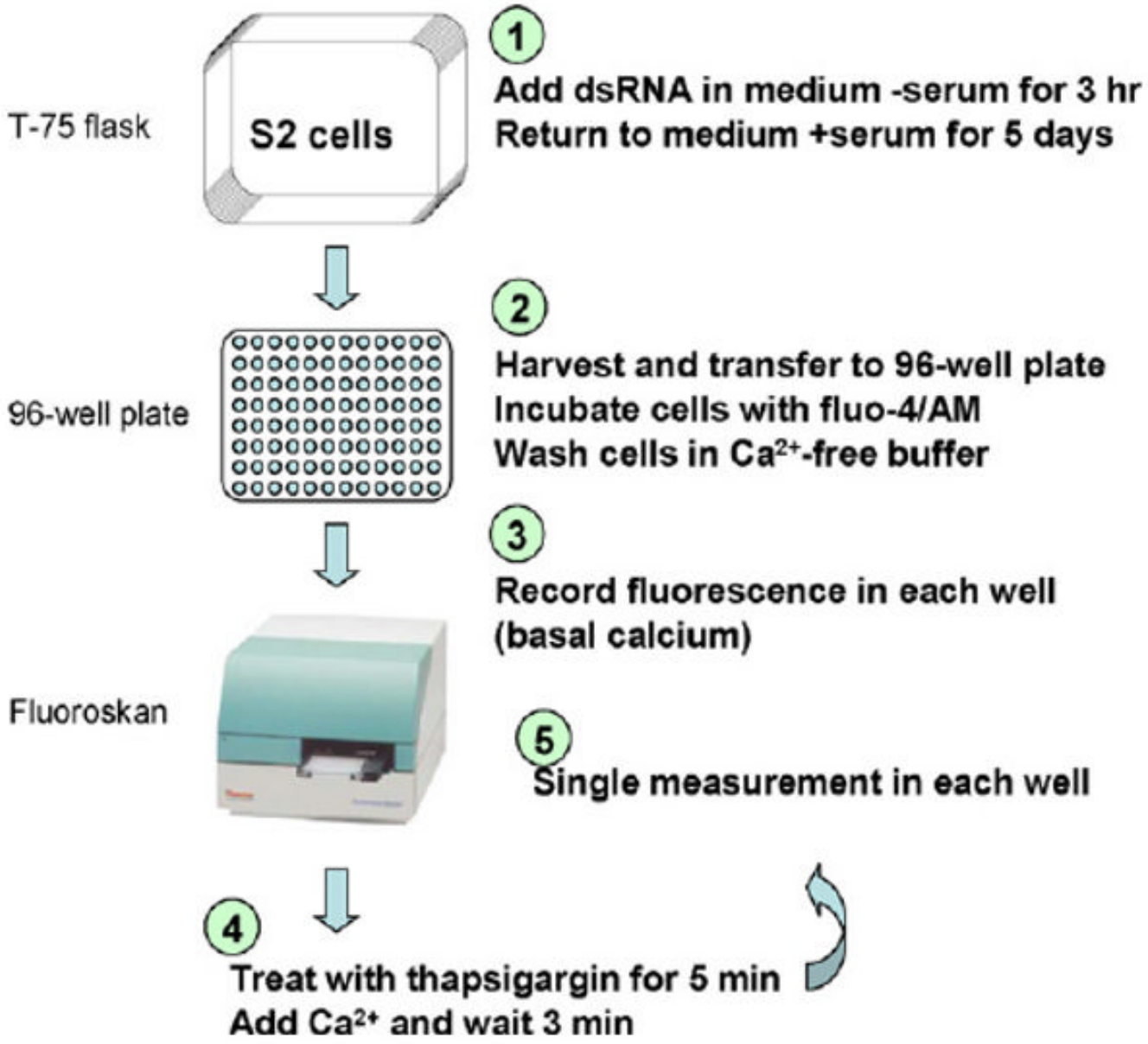

Fig. 2.
Candidate RNAi screening protocol.

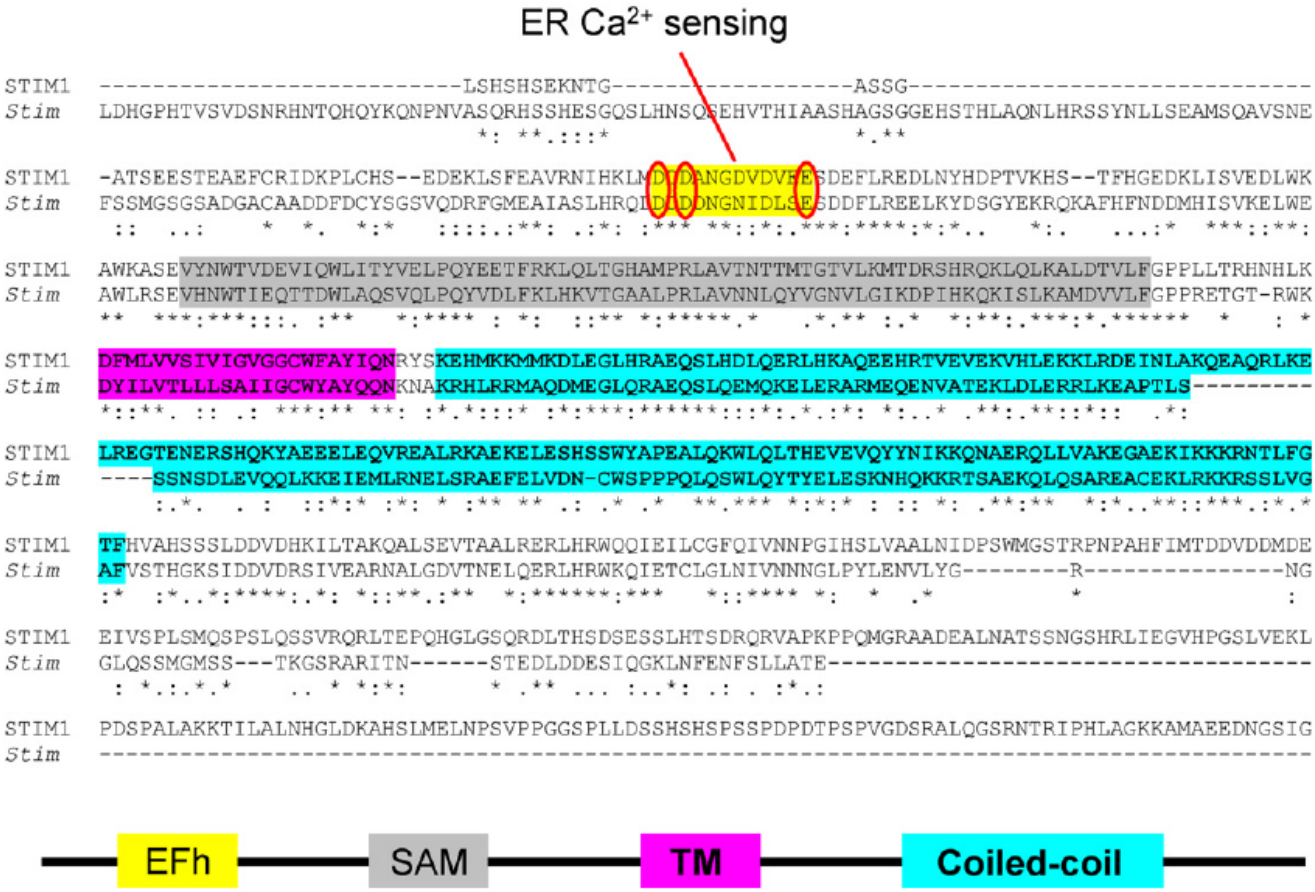

Fig. 3.
Stim1 sequences and motifs.

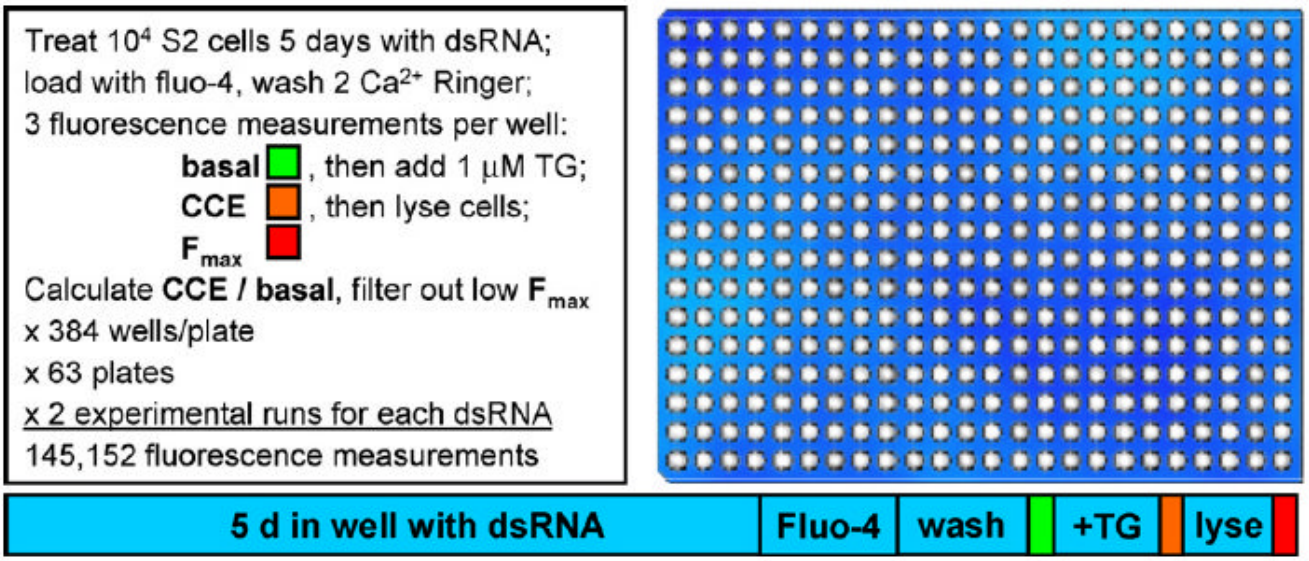

Fig. 4.
Genome-wide RNAi screening protocol.

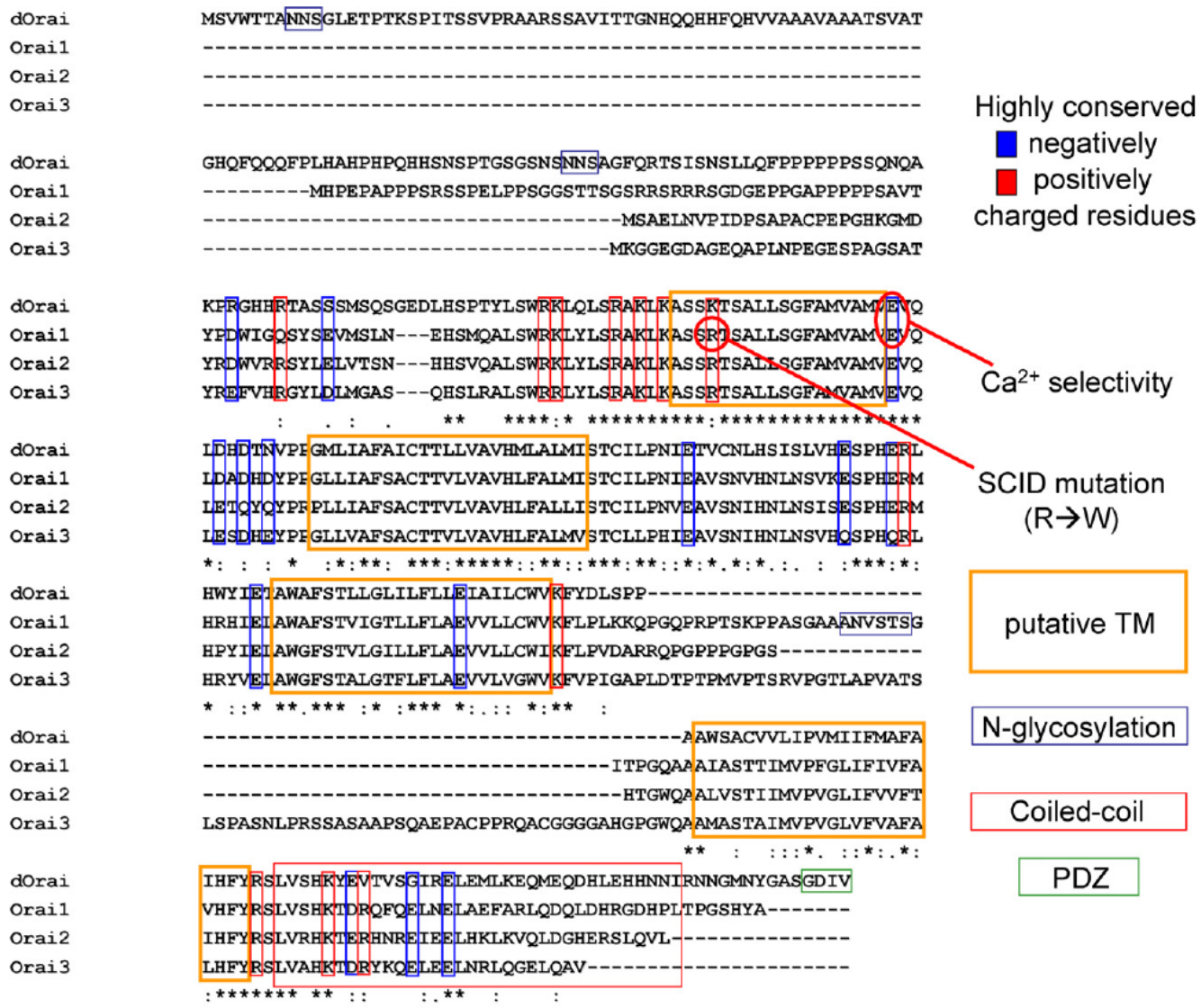

Fig. 5.
Fly and human Orai-related sequences and motifs.

Table 1

Comparison of CRAC current biophysical properties

Property	Native T cell CRAC [7,9–11,53–55]	Native S2 CRAC [26, 33]	Stim + Orai [33,48]	Stim + E180D Orai [48]
Store dependence	Yes	Yes	Yes	Yes
Inactivation by:				
Store refilling	Yes	Yes	Not tested	Not tested
Local $[Ca^{2+}]_i$	Yes	Yes	Yes	Yes
Run-down	Yes	Yes	Yes	Yes
Fast inactivation (100 ms time scale)	Yes	No	No	No
Calcium selectivity	Yes	Yes	Yes	No
Permeant divalent cations	$Ca^{2+} > Ba^{2+} > Sr^{2+} » Mg^{2+}$	$Ba^{2+} > Ca^{2+} > Sr^{2+} » Mg^{2+}$	$Ca^{2+} \sim Sr^{2+} \sim Ba^{2+} » Mg^{2+}$	Negligible
Unitary channel conductance in 20 mM Ca^{2+}	21 fS	36 fS	Not tested	N/A
Monovalent current in zero divalent	Yes	Yes	Yes	Yes
Monovalent cation selectivity, P_{Cs}/P_{Na}	$Na^+ > Cs^+$, 0.09–0.13	$Na^+ > Cs^+$, 0.17	$Na^+ > Cs^+$, 0.13	$Na^+ \sim Cs^+$, 0.71
Depotentiation	Yes	Yes	Yes	No
Pharmacology:				
Gd^{3+} or La^{3+}	nM (Gd^{3+}), reversible	nM (Gd^{3+}), reversible	nM (Gd^{3+}), reversible	nM (Gd^{3+}), reversible
SKF 96365	μM, partially reversible	μM, partially reversible	Not tested	Not tested
2-APB	Low μM – potentiation; high μM – inhibition	Low μM – potentiation; high μM – inhibition	Low μM – potentiation; high μM – inhibition	Low and high μM – complex behavior (potentiation and inhibition phases)

Results are summarized from the indicated references.