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CRAC channel-based optogenetics

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Abstract

Store-operated Ca^{2+} entry (SOCE) constitutes a major Ca^{2+} influx pathway in mammals to regulate a myriad of physiological processes, including muscle contraction, synaptic transmission, gene expression, and metabolism. In non-excitable cells, the Ca^{2+} release-activated Ca^{2+} (CRAC) channel, composed of ORAI and stromal interaction molecule (STIM), represents a prototypical example of SOCE to mediate Ca^{2+} entry at specialized membrane contact sites (MCSs) between the endoplasmic reticulum (ER) and the plasma membrane (PM). The key steps of SOCE activation include the oligomerization of the luminal domain of the ER-resident Ca^{2+} sensor STIM1 upon Ca^{2+} store depletion, subsequent signal propagation toward the cytoplasmic domain to trigger a conformational switch and overcome the intramolecular autoinhibition, and ultimate exposure of the minimal ORAI-activating domain to directly engage and gate ORAI channels in the plasma membrane. This exquisitely coordinated cellular event is also facilitated by the C-terminal polybasic domain of STIM1, which physically associates with negatively charged phosphoinositides embedded in the inner leaflet of the PM to enable efficient translocation of STIM1 into ER-PM MCSs. Here, we present recent progress in recapitulating STIM1-mediated SOCE activation by engineering CRAC channels with optogenetic approaches. These STIM1-based optogenetic tools make it possible to not only mechanistically recapture the key molecular steps of SOCE activation, but also remotely and reversibly control Ca^{2+}-dependent cellular

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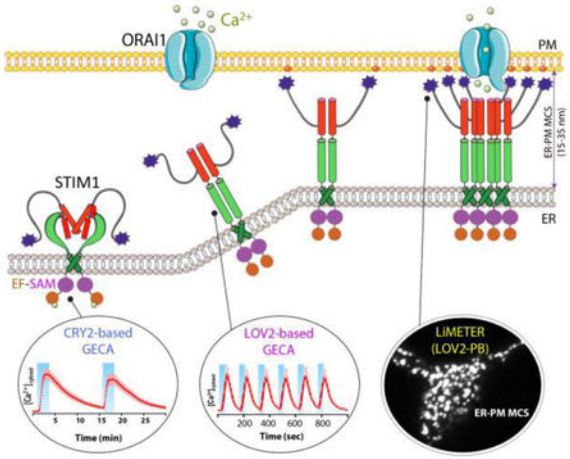
Conflicts of interest

The authors declare no competing financial interests.

processes, inter-organellar tethering at MCSs, and transcriptional reprogramming when combined with CRISPR/Cas9-based genome-editing tools.

Graphical Abstract:



Keywords

Optogenetics; Ion channel; ORAI; Stromal interaction molecule; CRISPR/Cas9; Membrane contact sites

1. STIM1 and ORAI1 as two major players in the SOC field

Calcium ions (Ca^{2+}) participate in various aspects of cellular activity and act as versatile chemical signals to control a myriad of biological processes, ranging from short-term responses such as muscle contraction, exocytosis, and synaptic transmission, to long-lasting effects on gene expression, metabolism, cell division, and cell death [1, 2]. The fact that binding Ca^{2+} to its targets can alter local electrostatic fields and trigger changes in protein conformation makes it a well-suited second messenger for signal transduction [3, 4]. Ca^{2+} influx is mediated by distinct Ca^{2+} channels, including voltage-gated, ligand-gated, store-operated and second messenger-operated channels [1, 2]. In excitable cells such as neurons and cardiomyocytes, extracellular Ca^{2+} flows into the cytosol through the activation of voltage-gated Ca^{2+} channels [5, 6], whereas in non-excitable cells, store-operated Ca^{2+} entry (SOCE) constitutes a major route of Ca^{2+} influx from the extracellular space into the cytoplasm [7-13]. SOCE is regarded as a unique pathway that connects the Ca^{2+} store depletion in the endoplasmic reticulum (ER) with the subsequent opening of highly selective Ca^{2+} channels in the plasma membrane (PM) [7]. SOCE is best exemplified by the Ca^{2+} release-activated Ca^{2+} channels (CRAC) that are initially characterized in cells of the immune system [14]. Rare gene alleles that compromise CRAC channel expression or activation (e.g., R91W) can lead to severe combined immunodeficiency in human patients [15-18]. Furthermore, gain-of-function mutations in the CRAC channel complex are regarded as the genetic culprits for Stormorken syndrome and tubular aggregate myopathy

[19-21]. Aberrant CRAC channel activity is also implicated in tumorigenesis, cancer metastasis, and cardiovascular disorders [13, 22, 23].

Store-operated CRAC channels are composed of a class of four-pass transmembrane proteins known as ORAI (named after the keepers of heaven's gate in Greek mythology; with three homologs ORAI1, ORAI2, and ORAI3), which function as pore-forming subunits [15, 24, 25]. ORAI proteins are directly gated by type I single-pass transmembrane proteins termed stromal interaction molecules (STIM1 and STIM2) [26-28]. As an ER-resident Ca^{2+} sensor and activator of ORAI Ca^{2+} channels, STIM1 contains an ER-luminal domain, a single transmembrane domain (TM), and a cytoplasmic domain (CT). The ER-luminal domain, consisting of EF-hand motifs and a sterile alpha motif (SAM) domain, is responsible for sensing ER Ca^{2+} fluctuation and initiating Ca^{2+}-depletion induced oligomerization [29, 30]. The TM domain transduces ER luminal signals [31, 32] toward the cytoplasmic domain that comprises a putative coiled-coil region (CC1), a minimal ORAI-activating region (SOAR or CAD or OASF; see *abbreviations*) [33-35], a Pro/Ser-rich region, an EB1-binding S/TxIP motif, and a C-terminal polybasic (PB) tail (Figure 1A). Following the identification of these two protein families as the molecular identities of the two-component CRAC channel complex, the critical steps and regulatory mechanisms involved in coordinating the exquisite molecular choreography of SOCE have been largely worked out through collective efforts from multiple groups (Figure 1B) [10-13, 36-38].

SOCE activation involves a dynamic intermembrane coupling between STIM1 and ORAI1 that culminates at ER-plasma membrane contact sites (MCSs), a specialized subcellular structure that is separated by a distance of approximately 15-35 nm [39, 40]. When the ER Ca^{2+} store remains full at rest, the full-length STIM1 presumably exists as a dimer or a lower-order oligomer maintained by its cytosolic region [41-44]. The Ca^{2+}-bound STIM1 luminal domain, when isolated in vitro by itself, stays as a monomer, with the aggregation-prone SAM domain fitting into the hydrophobic EF-hand cleft to form a compact structure [30, 45]. At the cytosolic side, the intramolecular coiled-coil clamp formed through CC1-SOAR interactions locks the cytoplasmic domain of STIM1 (STIM1-CT) in an inactive conformation [31, 32, 46], thereby preventing SOAR/CAD/OASF from encountering ORAI channels that would consequently lead to a constitutive Ca^{2+} influx [33-35]. Upon ER Ca^{2+} store depletion, Ca^{2+} release from the ER Ca^{2+} store leads to the dissociation of Ca^{2+} from EF-hand motifs, which induces EF-SAM oligomerization and stabilizes the conformation as an entropy-favored transition [29, 30]. Subsequently, the ER luminal signals are transmitted toward the STIM1-CT via the rearrangement of the TM domains [32], which prompts a conformational switch in the cytosolic juxta-membrane region and weakens CC1-SOAR association [31, 32, 46]. As a result, the minimal ORAI-activating domain, SOAR or CAD, is fully exposed and further oligomerized STIM1 molecules migrate toward the ER-plasma membrane contact sites to directly recruit and activate ORAI1 channels [33-35, 43]. Given the relatively weak strength of STIM1-ORAI1 interactions under physiological conditions, the redistribution of STIM1 towards the PM is further facilitated by the physical association between its own C-terminal PB domain with PM-embedded phophoinositides (PIs) [39, 44, 47, 48]. Sustained Ca^{2+} influx is required to further activate downstream effectors such as calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase that dephosphorylates a master transcription factor, the nuclear factor of activated T-cells (NFAT). This process ultimately

leads to NFAT nuclear import, which turns on key genes essential for lymphocyte activation [49, 50].

During the course of unraveling of STIM-ORAI coupling at ER-plasma membrane contact sites, a combination of traditional biochemical, pharmacological, structural and genetic approaches has been employed [30, 42, 51-56]. In this review, we aim to reconstruct the molecular choreography of SOCE by taking an unconventional optogenetic approach. Genetically-encoded photoswitches, such as the light-oxygen-voltage domain 2 (LOV2) from *Avena sativa* [57, 58] and cryptochrome 2 (CRY2) from *Arabidopsis thaliana* [59, 60], can be installed into STIM1 to mimic critical steps of CRAC channel activation, including STIM1 oligomerization, conformational switch, and ER-PM junction formation (Figures 1-3). We highlight herein the latest progress in this endeavor, with a concentration on the design and use of genetically-encoded Ca^{2+} actuators (GECAs; OptoSTIM1, Opto-CRAC, and BACCS) derived from STIM1 [61-63] and optical tethers (LiMETER [64] or OptoPBer [48]) that enable real-time photo-inducible ER-PM MCS assembly in living cells. We hope that this review will stimulate further thoughts on incorporating optogenetic approaches towards efforts to aid the mechanistic dissection of cell signaling and spur the development of next-generation optogenetic actuators for both research and therapeutic purposes.

2. CRY2-STIM1 chimeras to mimic inducible oligomerization of STIM1 luminal domain

Given that the dissociation of Ca^{2+} from the canonical EF-hand motif can trigger STIM1 oligomerization and cause CRAC channels activation [29], Luik *et al.* speculated that replacing the EF-SAM domain with a chemical-inducible dimerization system could similarly activate STIM1 to open ORAI channels without store depletion [53]. To test this hypothesis, they substituted the luminal Ca^{2+}-sensing domain of STIM1 with either the FKBP-rapamycin binding (FRB) domain or the FK506 binding protein (FKBP) [65]. Indeed, the addition of rapamycin prompted the rapid heterodimerization of FKBP-STIM1/FRB-STIM1 chimeric proteins, which was sufficient to drive STIM1 puncta formation and CRAC channel activation as seen with wild-type STIM1 upon store depletion [26-28]. This elegant study demonstrated the feasibility of bypassing store depletion to activate STIM1 with a chemical approach. In an independent study, Zhou *et al.* showed that covalently crosslinking STIM1-CT at residue 233 (where the cytosolic portion of STIM1 emerges from the ER membrane) to bring the N-terminus of CC1 region into close proximity could switch on STIM1-CT to adopt an active conformation [44]. These findings have paved the way for the employment of plant CRY2-based optical multimerizers to reversibly control STIM1 oligomerization and ultimately generate Ca^{2+} influx upon photostimulation.

Arabidopsis cryptochromes (AtCRY) belong to a family of evolutionarily conserved flavoproteins (with flavin adenine dinucleotide as chromophore; Figure 2A) that are homologous to DNA photolyases [66-68]. AtCRY2 controls photomorphogenesis in response to blue light in plants [68]. The activation of CRY2 is driven by light-induced electron transfer followed by flavin photoreduction [69]. CRY2, which rapidly undergoes monomer-to-oligomer clustering upon blue light illumination, has been successfully used to

manipulate protein oligomerization-dependent cell signaling [70, 71], including growth factor receptor activation and Ca^{2+} signaling. Compared to traditional chemical-based methods, the optogenetic approach offers two obvious advantages. First, the use of chemicals is associated with undesirable effects in living cells such as cytotoxicity and undesired perturbation to host cells due to potential off-target effects. Optogenetics can circumvent these caveats since only a simple pulse of visible light is needed to achieve similar functions. Second, the use of light makes it possible to manipulate signaling at a high spatial and temporal resolution and meet various kinetic requirements.

To enable the light-dependent oligomerization of STIM1, STIM1-CT (residues 238-685 [63] or 233-685 [72]) was fused after the N-terminal photolyase homology region of AtCRY2 (termed OptoSTIM1). When expressed in mammalian cells, OptoSTIM1 largely remained inactive in the dark but efficiently translocated toward the plasma membrane to evoke Ca^{2+} influx through endogenous ORAI channels upon blue light illumination ($t_{1/2,\ on}$: ~30-60 sec; $t_{1/2,\ off}$: ~5-6 min; Figures 1B). OptoSTIM1-mediated photoactivatable Ca^{2+} entry has been confirmed in a number of mammalian cells originating from different tissues (e.g., HeLa, HEK293, NIH3T3, COS-7 cells, astrocyte, human umbilical vein endothelial cells, and human embryonic stem cells), as well as in zebrafish embryos and the CA1 hippocampus of mice [63]. These findings, along with results obtained using the aforementioned chemical approaches [44, 53], have reinforced the notion that chemical or optogenetic manipulation is used to force close apposition of the juxtamembrane end of CC1, which likely phenocopies the consequence of Ca^{2+} depletion-induced EF-SAM luminal domain oligomerization, can activate STIM1 to open ORAI channels.

3. LOV2-STIM chimeras to mimic conformational switch within STIM1 cytoplasmic domain

The SOAR/CAD domain within STIM1-CT, when expressed alone, can fully engage and potently activate ORAI channels [33-35]. By contrast, longer STIM1-CT fragments, particularly those bearing the upstream CC1 region (residues 233-343), seem to act as substantially weaker ORAI activators [33-35]. This early observation led to the speculation that STIM1-CT might be kept in a quiescent state by self-caging the SOAR domain through an intramolecular autoinhibitory mechanism. Korzeniowski *et al.* claimed that a stretch of acidic amino acids within CC1 (residues 318-322; EEELE) might form electrostatic interactions with a polybasic region (residues 382-386; KIKKKR) in SOAR/CAD, thereby keeping the SOAR/CAD domain buried to prevent ORAI1 activation [73]. Neutralizing the acidic regions within CC1 (the 4EA mutant, EEELE>AAALA) was able to switch on STIM1-CT to cause a constitutive activation of SOCE [73]. Although the proposed physical contacts were not observed in a STIM1-ORAI1 complex structure that included both the acidic and basic regions of STIM1-CT (residues 312-387) [74], the notion of intramolecular conformational switch continued to resonate in the field and inspired follow-up studies to unveil the underlying molecular determinants [31, 32, 41, 44, 46, 75, 76].

To gain a more quantitative view on the conformational rearrangements within STIM1-CT, Muik *et al.* developed a fluorescence resonance energy transfer (FRET)-based biosensor that

detected the folding status of a STIM1-CT fragment (YFP-OASF-CFP; residues 233-474) [41]. Cells expressing the wild-type YFP-OASF-CFP exhibited robust FRET signals, suggesting that OASF adopts a relatively compact structure in close proximity to the donor and acceptor fluorophores in space. In contrast, the introduction of constitutively-activating STIM1 mutations into the initial segment of CC1 (L251S) or the ending helix of SOAR (L416S/L423S) led to a substantial reduction in FRET signals, implying the existence of an intramolecular clamp that locks STIM1 in an inactive conformation at rest [41]. This finding was further corroborated by an independent study using recombinant proteins, in which Zhou *et al.* used Tb^{3+}-based luminescence resonance energy transfer to demonstrate the existence of distinct conformations in WT and activated (L251S) STIM1-CT molecules [44]. Furthermore, artificial crosslinking of the CC1 domain could trigger STIM1-CT to adopt a more extended configuration [44]. These findings provide compelling evidence to support the conformational switch hypothesis underlying STIM1 activation. To map out key residues accounting for the intramolecular STIM1 autoinhibition, Ma *et al.* invented a two-component FRET-based assay by splitting STIM1 into two parts: Part I was composed of the EF-SAM-TM-CC1 domains that reside at the ER membrane (residues 1-310 or 1-342; with CFP attached to the C-terminus as donor), and Part II contained SOAR or its C-terminal extension variants (with YFP fused to the N-terminus as an acceptor) [31, 32]. When expressed alone, YFP-SOAR is known to be evenly distributed in the cytosol with partial decoration on the PM due to its interaction with endogenous levels of ORAI channels [33]. However, in the presence of part I, YFP-SOAR tightly docked toward the ER membrane, thereby confirming the interaction between CC1 and SOAR domains as part of the autoinhibitory mechanism proposed in earlier studies [41, 44, 73]. Through a series of truncation and deletion studies, Ma *et al.* further delineated key minimal regions to support the physical existence of a coiled-coil clamp, which involved the juxtamembrane CC1 region (residues 248-261) and the ending helix of SOAR (residues 408-437). This finding was further supported by FRET results from a complementary study by Fahrner *et al.*, who employed a so-called FIRE (FRET Interaction in a Restricted Environment) assay to dissect key structural elements mediating the CC1-SOAR interaction *in cellulo* [46]. Overall, these studies have yielded mechanistic insights into the structural determinants that govern STIM1 conformational switch during SOCE activation.

Inspired by the autoinhibitory property of CC1, He *et al.* attempted to use a genetically-encoded photoswitch derived from *Avena sativa* phototropin 1, the LOV2 domain [57, 58], to cage the minimal ORAI-activating region (Figure 2B). AsLOV2 has been extensively used to control protein actions because it undergoes remarkable conformational changes upon blue light stimulation. The AsLOV2 module consists of a flavin mononucleotide (FMN)-binding core domain known as a Period-ARNT-Single (PAS core) motif [77] and a C-terminal Jα helix (Figure 2B). A flavin chromophore is an endogenous cofactor that is widely accessible in mammalian cells, thus obviating the need of supplementing exogenous factors. Effector domains can be conveniently appended after the Jα helix of the AsLOV2 domain. In the dark, the Jα helix tightly docks to the PAS scaffold, thereby imposing potential steric hindrance to mask the active site or binding interfaces of the fused effector. Upon blue light illumination, photoexcitation creates a covalent adduct between the LOV2 residue C450 and the cofactor FMN, allowing the undocking of the Jα helix with subsequent

exposure of the effector domain [57, 78]. Solution NMR measurements have been conducted to estimate the free energy alterations between the dark and lit states [78]. The available free energy in a LOV2 photoswitch is estimated to be ~3.8 kcal mol^{-1}, which is sufficient enough to induce allosteric changes in polypeptides [79].

In the initial engineering of the LOV2-based GECAs, LOVS1K was constructed by fusing a STIM1-CT fragment (residues 233-450; including both the CC1 and SOAR domains) immediately after the C-terminal Jα helix [80]. LOVS1K was able to generate both global and local Ca^{2+} signals in response to blue light stimulation. This construct, nonetheless, was found to exhibit non-negligible dark activity. It led to constitutive Ca^{2+}-dependent NFAT nuclear translocation even when shielded from light [63], thereby preventing further applications in physiological processes that demand more precise control of Ca^{2+} signaling. Given that LOV2 and CC1 might compete for the same binding interface of SOAR and hence reduce the caging efficiency, He *et al.* decided to use STIM1-CT fragments without the CC1 region. After screening dozens of constructs by optimizing the LOV2-Jα helix, STIM1-CT fragments, and the linker in between, they eventually evolved a photoactivatable LOV2-STIM chimera ($LOV2_{404\text{-}546}$ + $STIM1_{336\text{-}486}$; designated Opto-CRAC) with a higher dynamic range of Ca^{2+} response while minimizing the dark activity [61]. This construct could rapidly translocate between the cytosol and the PM to reversibly induce Ca^{2+} influx following repeated dark-light cycle treatments ($t_{1/2,\ on}$ = ~8 s; $t_{1/2,\ off}$ = ~ 20 s; Figure 1B) [61]. Substitution of the human STIM1-CT fragment with a homologous region from zebrafish led to further reduction in background activity without sacrificing the high dynamic range of light-induced Ca^{2+} response [81]. In a third study, Ishii et al. employed a similar strategy to develop a set of tools known as BACCS (*B*lue light-*A*ctivated *C*a^{2+} *C*hannels Switch), composed of $LOV2_{404\text{-}538}$ and $STIM1_{347\text{-}448}$, as well as the corresponding *Drosophila* version dmBACCS2 that exhibited more potent ORAI-activating capability [62]. The latter two studies presented further diversified variants by generating PM-tethered or dimeric, tandemly-linked versions of LOV2-STIM1 chimeras, as well as fusion with ORAI1 to enable optogenetic control of Ca^{2+} influx in cells or tissues with low or no expression of endogenous ORAI channels [61, 62]. To confer the tightest caging of SOAR in the dark state, Nguyen *et al.* developed the second generation Opto-CRAC constructs by further appending Zdark (Zdk) downstream of STIM1-CT fragments. Zdk is a family of engineered affibodies that preferentially recognizes LOV2 in its dark state but dissociates from LOV2 in the presence of blue light [82]. Thus, the introduced Zdk can serve as an additional “lock” to further block the activity of SOAR to reduce the background activation [81].

The successful engineering of LOV2-STIM1 chimeras to photo-control Ca^{2+} flux furnishes an unambiguous piece of evidence to support the conformational switch hypothesis underlying STIM1 activation. Compared to CRY2-based GECAs, LOV2-STIM1 chimeras exhibit faster deactivation kinetics (~30 sec *vs.* ~300 sec) with comparable potency in photo-activating Ca^{2+} entry, thus making them most ideal for mimicking Ca^{2+} oscillations evoked by physiological stimulations [61]. LOV2-based GECAs have been rigorously tested in a variety of immortalized cell lines and primary cells derived from the nervous and immune systems [61, 62].

4. LiMETER for reversible control of MCS assembly

Membrane contact sites (MCS) are ubiquitous subcellular structures in eukaryotes and serve as the platform for Ca^{2+} signaling, lipid exchange, organelle fission and inter-organellar communications [39, 40, 83-85]. The initial report of MCS between ER/SR and the plasma membrane can be dated back to the observation of the so-called “triads” or “dyads” in excitable cells by electron microscopy [86]. Triads and dyads are formed by PM-localized voltage-gated Ca^{2+} channels and the SR-resident ryanodine receptors. They constitute the molecular basis for excitation-contraction coupling, converting changes in PM action potentials into Ca^{2+} release from the sarcoplasmic reticulum [87]. The gap distance in triads is estimated to be 9 to 12 nm [39, 88]. In comparison, in non-excitable cells, a fraction of ER-PM contact sites is marked by STIM and ORAI proteins during SOCE activation, with the gap distance determined to be 15 to 35 nm.

As described above, an activated STIM1 molecule translocates to ER-PM junctions and recruits ORAI molecules in the plasma membrane to mediate Ca^{2+} influx (Figure 1B). Notably, STIM1 redistribution toward the PM is facilitated by the interaction between the exposed C-terminal PB domain and the negatively-charged PIs in the inner leaflet of the PM [39, 44, 89-91]. Deletion of the PB domain (residues 672-685) prevents STIM1 translocation to form puncta at ER-PM junctional sites after Ca^{2+} store depletion despite intact self-oligomerization of STIM1 in the ER [34, 92]. To recapitulate the MCS assembly process in living cells, Jing *et al.* developed an optogenetic tool (designated LiMETER for *li*ght-*i*nducible *m*embrane *t*ethered peripheral *ER*) by using STIM1 as the engineering scaffold [48, 64]. In the prototypical design, LiMETER was inserted into the ER membrane by keeping the signal peptide and the single transmembrane domain from STIM1, while the Ca^{2+}-sensing and oligomerizable EF-SAM domain was replaced by a fluorescent protein (Figure 3A-B). On the cytosolic side, only the PB domain in the C-tail was retained, and the CC1-SOAR region was substituted with spacers of varying lengths and LOV2. In the dark, the PB domain was shielded because of the steric hindrance imposed by the upstream LOV2 domain. Upon blue light illumination, the PB domain was exposed to restore its PI-interacting capability. Therefore, LiMETER can be expressed in mammalian cells to reversibly label and manipulate the formation of ER-PM MCSs within seconds ($t_{1/2,\ on}$ = 14 s; $t_{1/2,\ off}$ = 25 s) [48]. This result clearly attests to the critical role of STIM1-PB in mediating STIM1-PI interaction, which efficiently facilitates the targeting of STIM1 toward PM.

LiMETER can be used as a scaffold to pinpoint residues that are essential for protein-PI interactions in living cells without the purification of recombinant proteins. Polypeptide sequences predicted to interact with PM-embedded PIs, such as $PI(4,5)P_2$ and $PI(3,4,5,)P_3$, can be conveniently grafted into the LiMETER construct, with subsequent introduction of mutations to unveil molecular determinants driving protein-PI associations. He *et al.* replaced the lysine residues of STIM1-PB with single or double alanine mutations to neutralize the positive charges of the PB domain. They found that single Lys-to-Ala mutations within STIM1-PB modestly decreased puncta formation. By contrast, the replacement of two adjacent lysine residues by alanine (e.g., K672A/K673A, K680A/K681A, or K684A/K685A) in the PB domain abolished photo-inducible puncta formation at

ER-PM junctions [48]. This site-directed mutagenesis study has provided an unequivocal line of evidence to support the indispensable role of these lysine residues in mediating protein-lipid association and facilitating the assembly of ER-PM MCSs.

LiMETER could further be repurposed as a molecular gauge to estimate the distance requirements between two organellar membranes. In the prototypical design of LiMETER without insertion of long spacers, the bridge connecting the ER and the PM spanned an estimated distance of 10 nm. ORAI1 molecules were completely excluded from the ER-PM contact sites marked by LiMETER puncta (Figure 3D, left). In contrast, when the inter-membrane gap distance was increased to above 25 nm via the introduction of repeated helical spacers (Figure 3C), ORAI1 molecules were able to freely diffuse into these contact sites (Figure 3E). This finding was in line with the observation that the intracellular regions of ORAI1 might project into the cytoplasm and require at least a gap distance of 10 nm. It also lends support to the tacit belief in the field that the efficient formation of STIM1-ORAI complexes during SOCE probably requires a distance of at least 25 nm (27.1 ± 2.8 nm) in the gap between the ER and the plasma membrane [93]. Collectively, the design of LiMETER to reversibly control the formation of ER-PM contact sites provides a generic engineering strategy to not only probe protein-lipid interactions in living cells, but also tune the intermembrane distance at a nanoscale level in order to manipulate the behavior of cell signaling proteins at high temporal and spatial resolution [48]. Ideally, optogenetic tools like LiMETER can be further exploited as a shuttle to deliver cargo into MCSs, which can aid in the dissection of molecular mechanisms governing the biogenesis, maintenance, and dynamic of membrane contact sites.

5. Applications of STIM1-based optogenetic tools

CRY2- and LOV2-based GECAs enable precise spatial and temporal control of Ca^{2+} signaling with tailored functions [72]. Compared to conventional microbial opsin-based optical tools [94], STIM1-derived GECAs offer higher specificity for Ca^{2+} and are more suitable for manipulating biological processes with relatively slower kinetics (seconds to hours) in non-excitable tissues. These tools have been successfully applied to faithfully phenocopy a variety of Ca^{2+}-modulated cellular events and physiological processes (Figure 4). Both CRY2-STIM1ct and LOV2-SOAR can be used to photo-induce Ca^{2+} influx in various cell types, including T cells, macrophages, dendritic cells, neurons, embryonic stem cells, and cancer cell lines. These cells are derived from different tissues and species as well, including human, mice, zebrafish, fruit flies, and worms [61-63]. Even though the light-triggered magnitude of Ca^{2+} signals seems to be heterogeneous in different cells due to the varied expression levels of endogenous ORAI channels, STIM1-based GECAs have been used to control hallmark Ca^{2+}-dependent physiological responses (Figure 4B). For instance, both OptoSTIM1 and Opto-CRAC were expressed in model cellular systems to drive the expression of Ca^{2+}/NFAT-dependent genes [61-63]. The induction of NFAT-mediated gene transcription, such as NFAT-dependent luciferase and insulin expression in HEK293T cells, indicates that Opto-CRAC has the capacity to photoactivate transfected cells via the cooperation of NFAT proteins with other transcriptional partners. As expected, transducing primary mouse $CD4^+$ T cells with Opto-CRAC led to a significant increase in the production of signature cytokines, such as IL2 and IFN-γ, in activated T cells [61]. Likewise, Human

THP-1-derived macrophages expressing Opto-CRAC secreted large quantities of IL-1β and processed caspase-1 in response to light stimulation, thus demonstrating the possibility of photo-tunable augmentation of inflammasome activation [61].

Apart from photo-manipulating hallmark Ca^{2+}/NFAT-dependent gene expression, Opto-CRAC has been further repurposed to control the expression of any endogenous genes (Figure 4). This was made possible by the invention of a light-inducible transcriptional reprogramming device that combines CRISPR/dCas9 with Opto-CRAC [81]. This synthetic device consists of two components. The first component contains a second-generation Opto-CRAC construct made of LOV2 and *Danio rerio* STIM1-CT (341-442) chimeras, which provide tighter control over Ca^{2+} signals than the first Opto-CRAC prototype. The second component, termed CaRROT (Figure 4A), consists of an N-terminal fragment of NFAT (residues 1-460) fused to dCas9 and transcriptional coactivators (VP64/VP160). Since the NFAT fragment used in CaRROT lacks a C-terminal DNA binding domain, it is unable to bind to endogenous NFAT targets. In the dark, CaRROT stays exclusively in the cytosol due to the phosphorylation of NFAT. Upon blue light illumination, Opto-CRAC induces Ca^{2+} influx from the extracellular space into the cytosol to trigger the dephosphorylation of NFAT and the subsequent nuclear entry of CaRROT. After entering the nuclei, dCas9-VP64 is directed by small guide RNAs (sgRNAs) to target sequences in the genome and turn on gene transcription. CaRROT thus allows for inducible transcriptional reprogramming. Theoretically, it can be further used in precision regenerative medicine by controlling the expression of genes that are critical for inducing the differentiation of induced pluripotent cells (iPSC) towards a defined cell fate. Likewise, CaRROT can also be reconfigured for the remote intervention of cancer progression. CaRROT, when combined with transcriptional repressors such as KRAB [95], can be repurposed to shut down the expression of oncogenes in cancer cells. In addition, when coupled with newly developed base editing tools [96], CaRROT can be used to correct mutations in the genome to reverse disease progression.

The afore-described *in vitro* data strongly supports the feasibility of using these GECAs for *in vivo* studies. However, the inability of blue light-absorbing photoreceptors to penetrate deep in tissues greatly limits the applications of these tools at organismal levels [97]. Neuroscientists often adopt a rather invasive approach to photoactivate microbial opsins by implanting invasive indwelling fiber optic probes into the brain. This protocol seems to be unfeasible with cells that are constantly circulating within the body. To tackle this challenge, He *et al*. first explored the use of lanthanide-doped upconversion nanoparticles (UCNPs) as a near-infrared (NIR) light transducer that could convert NIR light (980 nm) into blue light (470 nm) [98-100]. NIR light can penetrate at a depth greater than 2-3 cm [101-103]. When coupled with upconversion nanoparticles, the optogenetic operation window of Opto-CRAC could be shifted from the visible range to NIR wavelengths to enable wireless and remote photoactivation of Ca^{2+}-dependent signaling and optogenetic modulation of immunoinflammatory responses. In a mouse model of melanoma that used ovalbumin as a surrogate tumor antigen, Opto-CRAC in dendritic cells acted as a genetically-encoded “photoactivatable adjuvant” that improved antigen-specific immune responses and specifically destructed tumor cells [61]. A more recent proof-of-concept study has been used a similar approach to achieve deep brain stimulation in the central nervous system [104].

6. Summary and future directions

Overall, the optogenetic engineering of CRAC channels turns out to be an extremely rewarding journey, in that it not only yields insight into the working mechanism underlying STIM1-mediated SOCE activation but also affords unconventional tools to study cell physiology. STIM1-derived GECAs can faithfully recapture the key steps involved in STIM1-ORAI coupling at membrane contact sites to ultimately evoke Ca^{2+} influx from the extracellular space. As illustrated in the preceding sections, optogenetic tools engineered from STIM1 also open new opportunities for remotely interrogating Ca^{2+}-dependent biological processes, dynamically examining lipid-protein interaction and delivering proteins of interest to this specialized subcellular compartment, and screening protein and chemical modulators that are involved in maintaining and remodeling cell signaling at MCSs.

Aside from CRAC channels, more tools have been recently invented to control the activity of other types of Ca^{2+} channels, including TRPC channels [105] and voltage-gated Ca^{2+} channels [106]. Nonetheless, existing efforts are almost exclusively directed to engineer ion channel regulatory units or synthesize photo-sensitive chemical modulators. Direct engineering of a photoswitchable *bona fide* Ca^{2+} channel, like those done with channelrhodopsin [107] or potassium channels [108], is still missing in the literature. Capitalizing on naturally-evolved photosensory domains that can be installed into signaling proteins, we hope that optogenetic engineering approaches described above can be adopted to control ion channels *per se* and be further extended to manipulate other types of MCSs formed between the ER and various intracellular organelles, including mitochondria, lysosomes, endosomes, peroxisomes, and lipid droplets [39, 40, 83-85]. In the near future, we anticipate the continuing expansion of the optogenetic toolkit tailored for the Ca^{2+} signaling field with on-demand kinetics and high specificity. Finally, we hope that near-infrared light-stimulatable nanomaterials and optogenetic tools, which are expected to have deeper tissue penetration [61, 104, 109, 110], will further accelerate the translational application of optogenetic actuators *in vivo*.

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Abbreviations

ER	endoplasmic reticulum
PM	plasma membrane
MCS	membrane contact site
SOCE	store-operated calcium entry
CRAC	calcium release-activated calcium channel

STIM	stromal interaction molecule
CRY2	cryptochrome 2
LOV2	light-oxygen-voltage domain 2
PB	polybasic domain
SOAR	STIM1 Orai-activating region
CAD	CRAC activation domain
OASF	Orai-activation small fragment
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
LiMETER	light-inducible membrane-tethered peripheral ER
CaRROT	calcium-responsive transcriptional reprogramming tool

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Highlights

- CRY2-STIM1 chimeras to mimic STIM1 oligomerization for optical activation of ORAI
- LOV2-STIM1 chimeras to recapitulate STIM1 conformational switch
- LiMETER to manipulate protein-lipid interactions and MCS assembly
- STIM1-derived optogenetic tools to phenocopy hallmark Ca^{2+}-dependent responses
- Rewiring photo-inducible Ca^{2+} signaling for precise transcriptional reprogramming

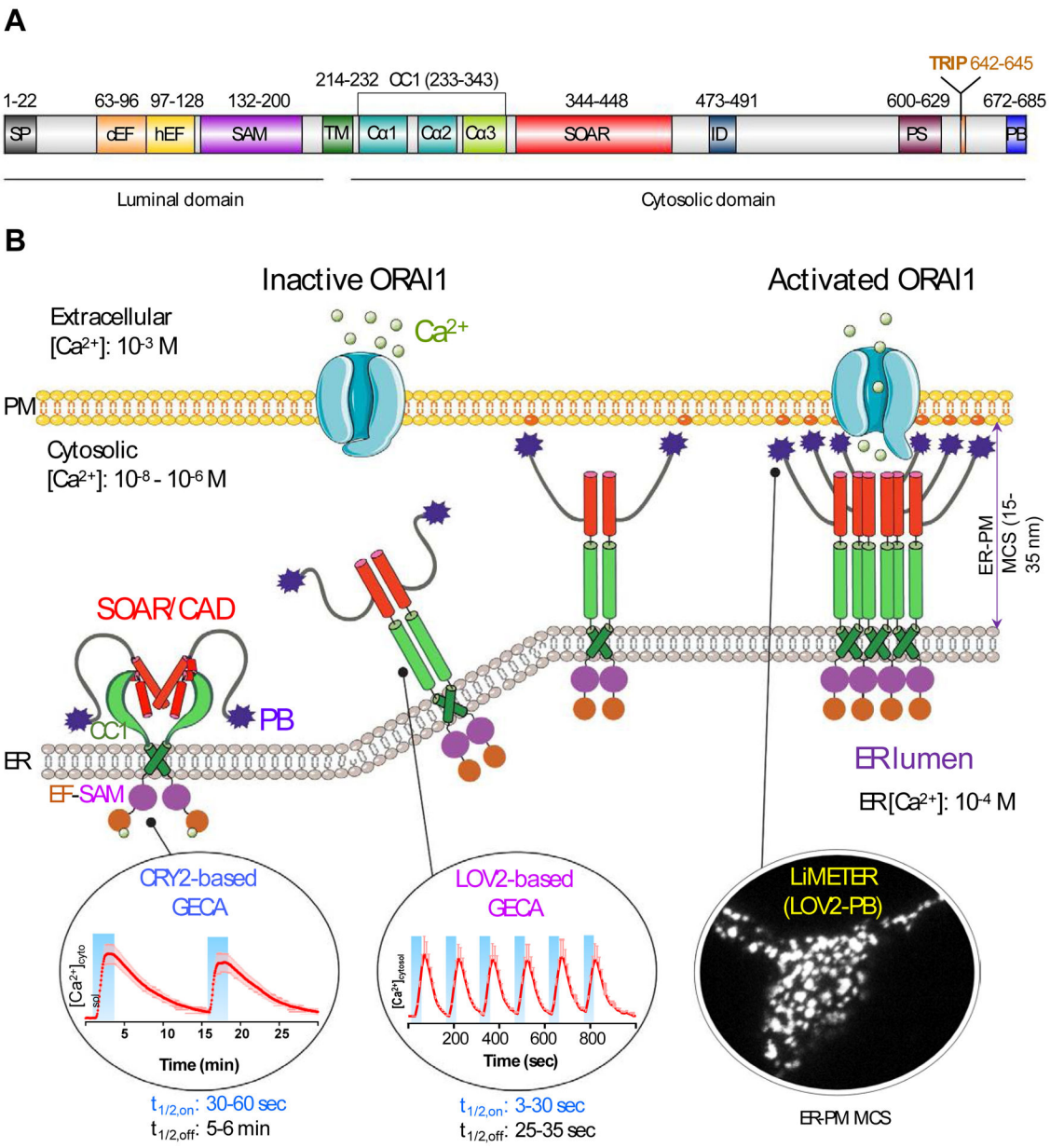

Figure 1 |. Engineering STIM1 to convert SOCE at ER-PM membrane contact sites (MCSs) into light-operated Ca^{2+} entry.

(A) Schematics of the domain architecture of human STIM1. The luminal domain of STIM1 contains a signal peptide (SP), a hidden non-Ca^{2+} binding EF-hand motif (hEF), a canonical Ca^{2+} binding EF-hand motif (cEF), and a sterile alpha motif domain (SAM). The cytoplasmic domain comprises a putative coiled-coil region (CC1), a minimal STIM1 ORAI-activating region (SOAR or CAD or OASF), an inactivation domain (ID), a proline/serine-rich region (PS), an EB1-binding sequence (TRIP), and a C-terminal polybasic tail (PB).

(B) Cartoon illustration of the dynamic coupling between STIM1 and ORAI1 during SOCE activation at ER-PM apposition, a specialized membrane contact site that is separated by a

distance of approximately 25-35 nm without membrane fusion. The major steps in this tentative model include: (i) Ca^{2+} depletion induces oligomerization of the luminal EF-SAM domain to initiate STIM1 activation; (ii) the luminal signal is transduced toward the cytoplasmic side to overcome STIM1 autoinhibition mediated by the intramolecular CC1-SOAR interaction, thereby triggering a conformational switch to expose SOAR/CAD/OASF and the C-terminal PB domain; (iii) activated STIM1 undergoes further oligomerization and its subsequent migration toward the PM is facilitated by the association between the positively charged PB domain and PM-embedded, negatively-charged phosphoinositides. SOAR/CAD/OASF is responsible for directly engaging and activating ORAI channels to mediate Ca^{2+} flux from the extracellular space into the cytosol. When coupled to plant-derived photosensory domains, such as cryptochrome 2 (CRY2) and light-oxygen-voltage domain 2 (LOV2), these critical steps can be individually mimicked with engineered Opto-CRAC constructs (CRY2-STIM1 chimeras or LOV2-STIM1 chimeras) and ER-tethered LOV2-PB proteins (designated LiMETER for light-inducible membrane-tethered peripheral ER). Representative Ca^{2+} signals generated by Opto-CRAC variants following repeated light-dark cycles are shown with the circles, along with a typical image showing the light-induced assembly of ER-PM MCSs in HeLa cells revealed by total internal reflection fluorescence (TIRF) microscopy. These constructs are available at Addgene (accession ID: #101245, #101246, #113933 and #113934).

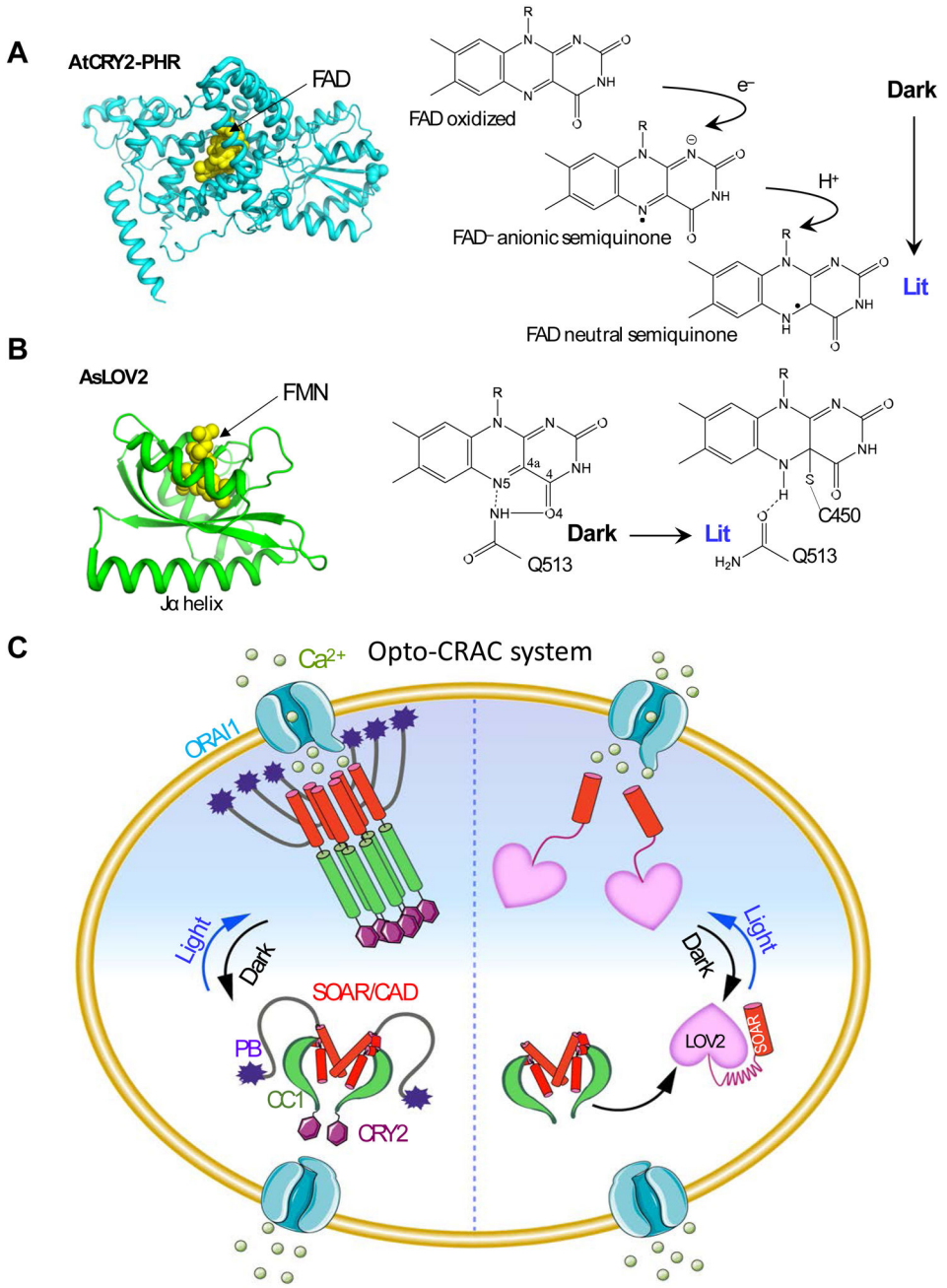

Figure 2 |. Opto-CRAC for optical control of Ca^{2+} influx in mammalian cells.

(A-B) The 3D structures and simplified photocycle reactions of AtCRY2-PHR (modeled from CRY1; PDB entry: 1U3D; **A)** and AsLOV2 (PDB entry: 2V0W; **B)**. FAD, flavin adenine dinucleotide; FMN: flavin mononucleotide.

(C) The left semi-circle depicts an Opto-CRAC construct made of a CRY2-STIM1 fusion. Upon light illumination, CRY2 undergoes oligomerization, mimicking Ca^{2+}-depletion induced EF-SAM multimerization, to trigger STIM1 activation with subsequent opening of ORAI Ca^{2+} channels situated in the plasma membrane. The right semi-circle illustrates the engineering strategy of an alternative form of Opto-CRAC, in which the CC1-SOAR

interaction-mediated intramolecular autoinhibition is recapitulated by LOV2-SOAR fusion in the dark. Following photostimulation, LOV2 undergoes rapid conformational changes to expose the C-terminal effector domain, thereby restoring the potent ORAI-activating activity of SOAR to elicit Ca^{2+} influx.

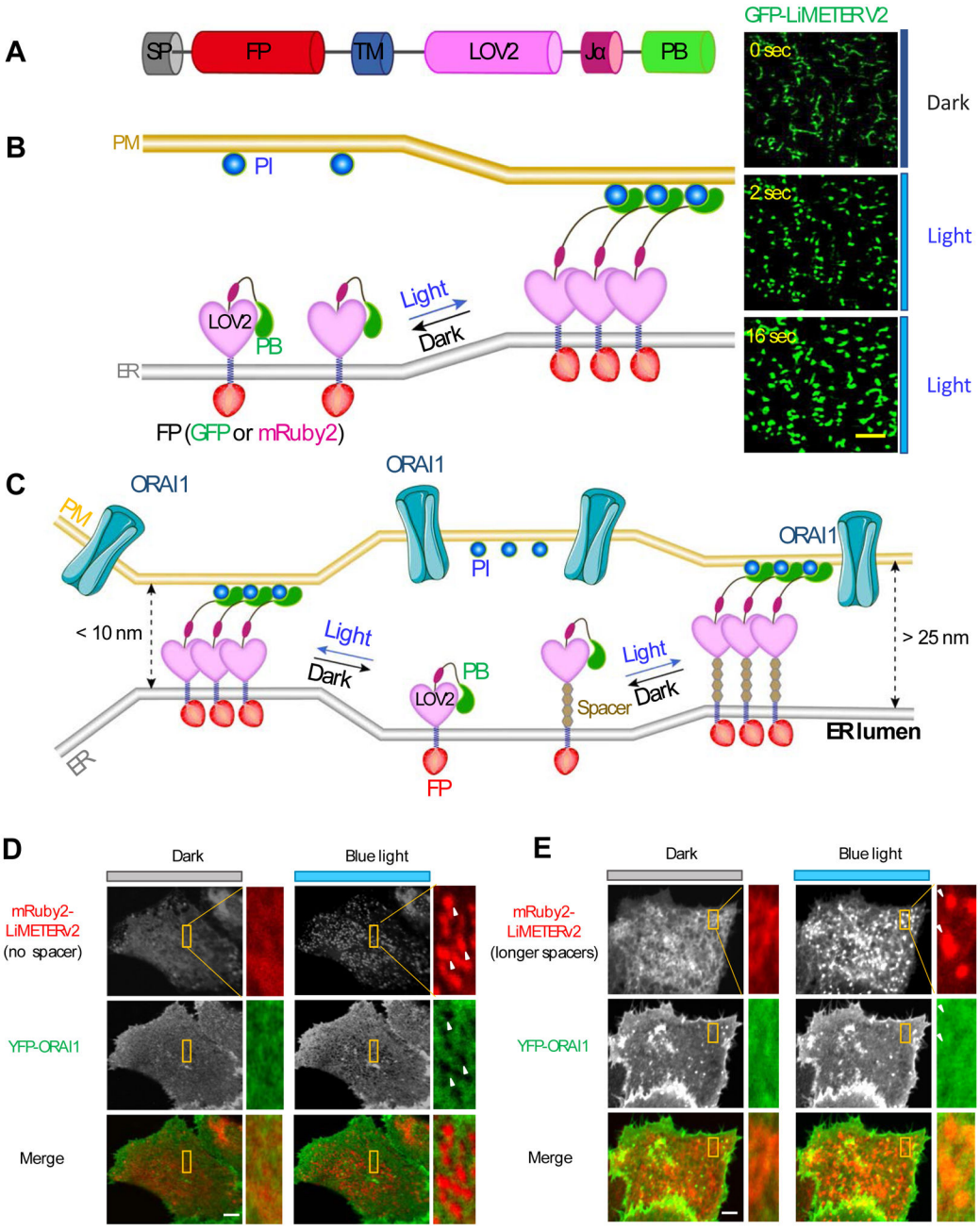

Figure 3 |. Mimicking reversible ER-PM MCS formation with LiMETER.

(A) LiMETER is engineered from STIM1 by replacing the luminal EF-SAM with a fluorescent protein (FP) and substituting the majority of its cytosolic domain with the LOV2 photosensitive module. The ER-targeting signal peptide (SP) and transmembrane domain (TM) were used to retain the synthetic protein to ER, whereas the C-terminal PB affords the tethering function through protein-phosphoinositide (PI) interaction. In the prototypical design, PB is derived from the Rit GTPase. In a recently improved version (also termed as OptoPBer or LiMETERv2), the PB is isolated from STIM1.

(B) Schematic representation of the working principle of LiMETER. In the dark, the Jα helix tightly docks to the core body of LOV2, thus imposing steric hindrance to the C-terminally fused PB domain to prevent its association with PM-resident PIs. Upon light illumination, the Jα helix unwinds and releases the constraints on PB, allowing itself to bind to the PIs to bridge the gap between ER and PM apposition. Shown on the right are typical images of GFP-tagged LiMETERv2 under a TIRF microscope following blue light irradiation.

(C) LiMETER can be engineered to manipulate the gap distance between ER-PM MCSs. Spacers with varying lengths can be modularly inserted between the TM and LOV2 domains to bridge a range of gap distances at MCSs. A gap distance of over 25 nm is required to allow efficient diffusion of ORAI1 proteins into MCS to evoke Ca^{2+} influx.

(D-E) Representative images of HeLa cells co-expressing YFP-ORAI (green) and mRuby-LiMETERv2 (red) with no spacer (D) or a long spacer (E). The yellow boxed regions were enlarged for a better visualization of the relative distribution of the two components in the same cell. YFP-ORAI1 was excluded from ER-PM MCSs (arrows) marked by mRuby-LiMETERv2 in the absence of the spacer (D). By contrast, YFP-ORAI1 can freely diffuse into ER-PM MCSs labeled by mRuby-LiMETERv2 with an appropriate spacer (E).

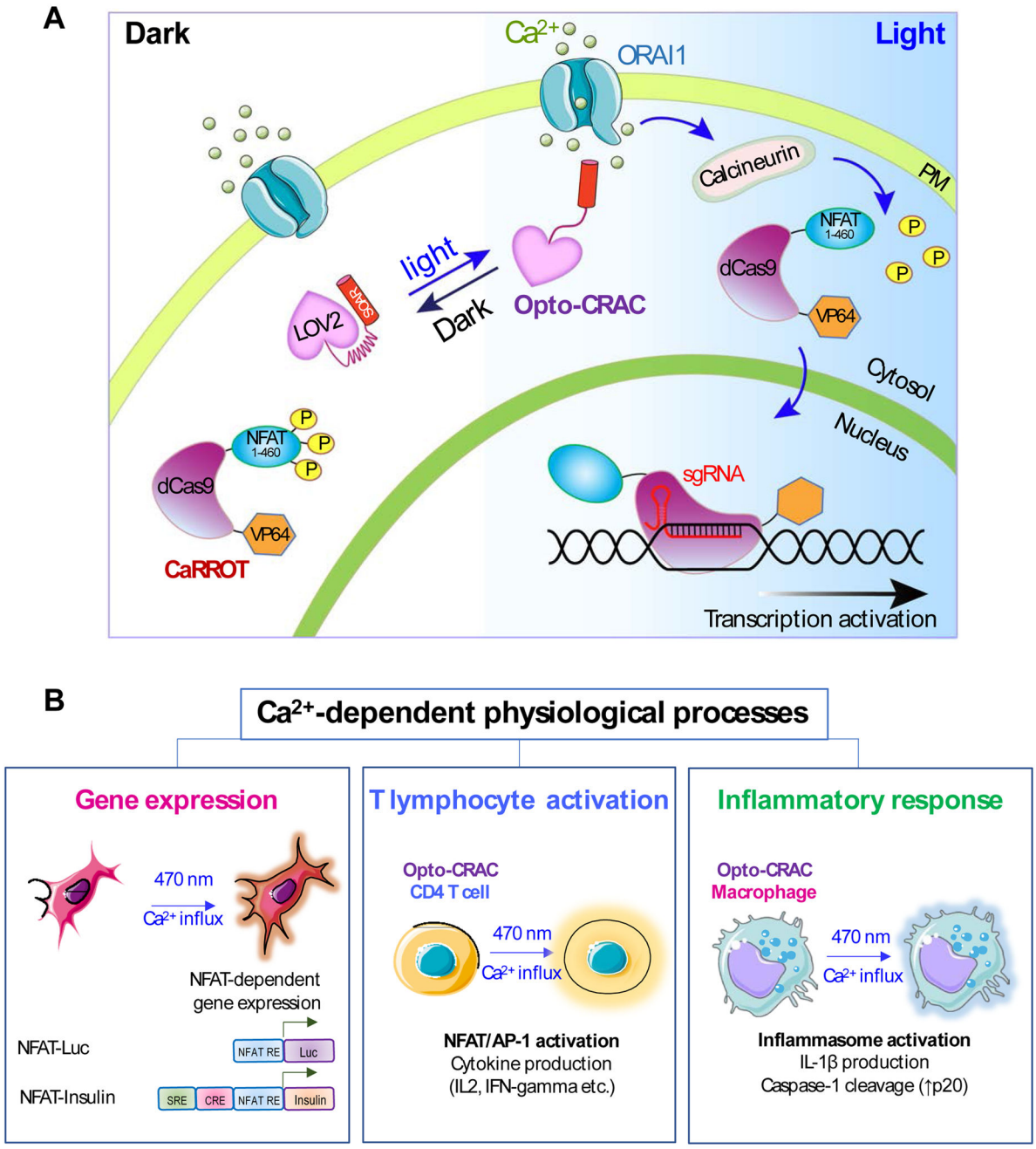

Figure 4 |. Examples illustrating potential applications of STIM1-derived optogenetic tools.

(A) Rewiring photoactivatable Ca^{2+} influx for light-inducible modulation of gene expression. Upon light stimulation, Opto-CRAC induces cytosolic Ca^{2+} influx to activate the Ca^{2+}/CaM-dependent phosphatase, calcineurin, which in turn dephosphorylates the N-terminal fragment of NFAT ($NFAT_{1-460}$) to drive the nuclear entry of a synthetic transcription regulatory device made of catalytically-dead Cas9 and the transcriptional coactivator VP64 ($NFAT_{1-460}$-dCas9-VP64; designated as CaRROT for Ca^{2+}-responsive transcriptional reprogramming tool). In the presence of small guide RNAs (sgRNAs), CaRROT can be precisely targeted to specific genomic loci to photo-tune the expression of

endogenous genes to achieve tailored function. Likewise, other effector domains such as transcriptional repressors (e.g., KRAB) and epigenetic regulators (DNA or histone modifying enzymes) [95, 111, 112] can be installed to replace VP64, thereby enabling photoswitchable inhibition of gene expression and epigenetic remodeling.
(B) Opto-CRAC can be applied to photo-control Ca^{2+}-dependent gene expression (left), cytokine production in T lymphocytes (middle), and inflammasome activation in macrophage (right). Optogenetic immunomodulation is likely to be of translational values for remote and personalized control of the immune system to fight invading pathogens and cancer with high precision.