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REVIEW

Molecular regulation of calcium-sensing receptor (CaSR)-mediated signaling

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Abstract

Calcium-sensing receptor (CaSR), a family C G-protein-coupled receptor, plays a crucial role in regulating calcium homeostasis by sensing small concentration changes of extracellular Ca^{2+}, Mg^{2+}, amino acids (e.g., L-Trp and L-Phe), small peptides, anions (e.g., HCO_3^- and PO_4^{3-}), and pH. CaSR-mediated intracellular Ca^{2+} signaling regulates a diverse set of cellular processes including gene transcription, cell proliferation, differentiation, apoptosis, muscle contraction, and neuronal transmission. Dysfunction of CaSR with mutations results in diseases such as autosomal dominant hypocalcemia, familial hypocalciuric hypercalcemia, and neonatal severe hyperparathyroidism. CaSR also influences calciotropic disorders, such as osteoporosis, and noncalciotropic disorders, such as cancer, Alzheimer's disease, and pulmonary arterial hypertension. This study first reviews recent advances in biochemical and structural determination of the framework of CaSR and its interaction sites with natural ligands, as well as exogenous positive allosteric modulators and negative allosteric modulators. The establishment of the first CaSR protein–protein interactome network revealed 94 novel players involved in protein processing in endoplasmic reticulum, trafficking, cell surface expression, endocytosis, degradation, and signaling pathways. The roles of these proteins in Ca^{2+}-dependent cellular physiological processes and in CaSR-dependent cellular signaling provide new insights into the molecular basis of diseases caused by CaSR mutations and dysregulated CaSR activity caused by its protein interactors and facilitate the design of therapeutic agents that target CaSR and other family C G-protein-coupled receptors.

KEYWORDS

calcium signaling, calcium-sensing receptor, G-protein-coupled receptors, structure, trafficking

Key points

- This review highlights recent efforts to understand CaSR, including biochemical and structural aspects, interaction sites with Ca^{2+} and amino acids, agonist/antagonist development, and activation mechanisms.
- The revelation of novel CaSR interactors influencing CaSR-related processes provides crucial insights into disease mechanisms and therapeutic strategies targeting CaSR and family C GPCRs.

Li Tian and Corey Andrews contributed equally to this work.

1 | CALCIUM DYNAMICS AND THE CALCIUM-SENSING RECEPTOR

Calcium ions (Ca^{2+}), as both first and second messengers, regulate important physiological activities such as neurotransmission, muscle cell contraction, gene transcription, apoptosis, and the blood clotting cascade.[1,2] To accomplish these many functions, Ca^{2+} signals manifest as oscillations and waves with highly regulated spatial-temporal Ca^{2+} dynamics. Depending on the stimuli and cell types, Ca^{2+} oscillations can be fast or slow with a frequency ranging from Hz in neurons to tens of mHz in nonexcitable cells[3] to trigger neurotransmitter release, gene expression, cellular growth, and fertilization.[4] It is important for each cell to have an array of regulatory mechanisms to tightly regulate the availability of Ca^{2+}, and complications that dysregulate these regulatory mechanisms are either responsible or partially liable for the pathogenesis of various Ca^{2+}-dependent diseases.

Concentration gradients and maintenance mechanisms of those gradients ensure the robustness of Ca^{2+} signaling. It is also possible that excessively high concentrations of Ca^{2+} can precipitate with anionic species such as phosphate (PO_4^{3-}) in the cytoplasm.[5–8] Ca^{2+}-dependent calnexin (CNX) cycle uses the molecular chaperone CNX to ensure that nascent peptides are preprocessed (i.e., N-glycosylated) and folded in the endoplasmic reticulum (ER) membrane before trafficking.[9] If there are abnormalities in the local concentrations of Ca^{2+} in the cytoplasm, the cell may also initiate the unfolded protein response.[10] Although extracellular Ca^{2+} ($[Ca^{2+}]_o$) concentration varies from 1.1 to 1.8 mmol/L, cytosolic Ca^{2+} concentrations ($[Ca^{2+}]$) range from ~100 nmol/L at baseline to 10 mmol/L at maximal stimulation. The ER Ca^{2+} stores range between 0.1 and ~1 mmol/L.[11–13] Ca^{2+} buffer proteins, such as calbindins, parvalbumin, and calmodulin (CaM) in the cytosol,[14] and calsequestrin[15] and calreticuliN (CRT)[16] in the ER/SR, maintain appropriate physiological concentrations of Ca^{2+}. Sensitivity to Ca^{2+} concentration changes enables regulation of many proteins by CaM[17,18] including glycogen phosphorylase,[18] CaM-dependent kinase II, enriched in nervous, cardiac, and muscular tissues,[19,20] and widely utilized CaM-dependent phosphatases, such as calcineurin,[21] which activate important immune transcription factors, such as the nuclear factor of activated T cells.[22]

Other regulatory mechanisms include Ca^{2+}-ATP pumps, such as SERCA[23] and PMCA,[24] and Na^+/Ca^{2+} exchange transporters that ensure both steady-state ER Ca^{2+} ($[Ca^{2+}]_{ER}$) and $[Ca^{2+}]_o$.[25] Membrane Ca^{2+} gradients and signaling are also regulated by channels, including inositol 1, 4, 5-trisphosphate (IP_3) receptor (IP_3R)[26] and the ryanodine receptor (RyR)[27] on the ER/SR membrane, the mitochondrial calcium uniporter on the inner mitochondrial membrane,[28] plasma membrane/sarcolemma integral cationic channels, such as the N-type, L-type, and T-type voltage-gated Ca^{2+} channels,[29] the transient receptor potential (TRP) family,[30] hyperpolarizing cyclic-nucleotide-sensitive cationic channels,[31] mechanoreceptive cationic channels, P2 purinergic cationic channels,[32,33] and arachidonate-regulated Ca^{2+} selective cationic channels.[34] Processes such as Ca^{2+}-induced Ca^{2+} release,[35] Ca^{2+}-induced Ca^{2+} entry,[35] store-operated Ca^{2+} entry,[36] and store-operated Ca^{2+} release[37] are regulated by the store-operated Ca^{2+} release-activated Ca^{2+} channels, whose Orai domains undergo a conformational change upon binding to the Ca^{2+}-sensing ER membrane protein stromal interaction molecule.[38]

The discovery of the parathyroid Ca^{2+}-sensing receptor (CaSR) by Dr. Edward Brown et al. in 1993 established a new paradigm of Ca^{2+} signaling.[39] Subsequent studies reported that $[Ca^{2+}]_o$ acts as a first messenger to regulate diverse cellular processes via CaSR and 14 other family C G-protein-coupled receptors (GPCRs), including metabotropic glutamate receptors (mGluRs)[40,41] and γ-aminobutyric acid ($GABA_B$) receptors; taste receptors 1 (T1R), 2 (T2R), and 3 (T3R); the orphan receptor/osteocalcin receptor GPRC6; and the vomeronasal G-protein receptors.[42] Although primarily expressed in the parathyroid gland,[42] CaSR fulfills a central role in regulating extracellular Ca^{2+} homeostasis,[43] as well as many physiological and pathophysiological processes across multiple organs, including the parathyroid glands, kidney, bone, brain, heart, and skin. This regulation is largely orchestrated by its cooperative capability in response to small extracellular changes in Ca^{2+} concentration ($[Ca^{2+}]_o$) and pH as a function of bioavailable bicarbonate (HCO_3^-), as well as extracellular magnesium ($[Mg^{2+}]_o$), aromatic L-amino acids (e.g., L-Trp, L-Phe),[44] polyamines,[45–48] phosphate (PO_4^{3-}), and sulfate (SO_4^{2-}). CaSR can distinguish small Ca^{2+} fluctuations (<0.1 mmol/L) in the extracellular environment due to multiple binding sites on the CaSR extracellular domain (ECD) and the highly cooperative binding process with Ca^{2+} (Hill number ~3).[49–51] L-Phe, L-Trp, and arylhydrocarbon-containing amino acid derivatives act as endogenous co-agonists to potentiate functional activities.[52,53]

CaSR is a pleiotropic receptor, as it is coupled to several G-protein-regulated intracellular signaling pathways ($G_{q/11}$, $G_{i/o}$, G_s, and $G_{12/13}$).[54–56] CaSR activates phospholipase C (PLC)[57,58] to produce IP_3 by interacting with $G_{q/11.}$ This releases calcium from the ER to induce cytosolic calcium increase and calcium oscillations. The pattern of $[Ca^{2+}]_i$ oscillations is one of the most important signatures reflecting the state of CaSR activity.[59,60] Diacylglycerol (DAG) produced from PIP_2 further activates protein kinase C (PKC) to regulate protein phosphorylation cascades in cell survival, differentiation, and proliferation via mitogen-activate protein kinase (MAPK)[43,54,61,62] and the phosphorylated extracellular-signal-regulated kinase 1 (ERK1) and ERK2.

By interacting with $G_{i/o}$ proteins, CaSR inhibits the production of cAMP via adenylate cyclase. Dimeric CaSR displays signaling bias, depending on the types of agonists, drugs, and the cell/tissue types.[63] Downstream, the ATP-bound and the dissociated subunits of the heterotrimeric G-proteins regulate various signaling pathways, including phospholipase A_2 and its downstream prostaglandin E2 pathway;[57,64] PLC,[57,58] phospholipase D,[57] protein kinase A,[65] PKC, MAPK,[61] and Rho.[66] In turn, their activities are regulated by RGS proteins, such as RGS5, previously implicated in hyperparathyroidism,[67] which act as GTP-activated proteins for the GTP-bound α-subunit, thereby accelerating GTP hydrolysis[68] (Figure 1).

The parathyroid glands act on several organs through CaSR-mediated hormonal regulation. These organs include the small intestines, the proximal and distal tubules in the cortical kidney, and bone tissue. The small intestines absorb Ca^{2+} and PO_4^{3-} by vitamin D (i.e., calcitriol), which is activated via parathyroid hormone (PTH) stimulation of the tubule cells in the kidneys. Although blood concentrations of PO_4^{3-} and Ca^{2+} are dependent upon vitamin D-dependent absorption in the small intestine,[69] they are also dependent upon rate of deposition of Ca^{2+} by osteoblasts versus rate of resorption of Ca^{2+} by osteoclasts in bone tissue and also by reabsorption of PO_4^{3-} from the proximal tubule and Ca^{2+} from the distal tubule in the nephron. Secretion of PTH and calcitonin by the parathyroid glands regulate Ca^{2+} and PO_4^{3-} dynamics on a physiological level.[70,71] Extracellular Ca^{2+} in the blood stimulates the CaSR on chief cells in the parathyroid gland, resulting in decreased secretion of PTH.[72,73] Consequently, decreases in PTH secretion reduce the rate of bone resorption relative to the rate of bone deposition, thereby maintaining homeostasis blood concentrations of Ca^{2+} between 1 and 2 mmol/L and PO_4^{3-} at ~0.8 mmol/L.[71]

Alternatively, increased calcitonin release by the parathyroid glands is facilitated by increased stimulation of CaSR.[74] As expected, increased calcitonin release, as a consequence of CaSR activation in the parathyroid, increases bone deposition of Ca^{2+} and PO_4^{3-}. CaSR activation also decreases vitamin D activation in the kidney, which in turn reduces intestinal absorption of Ca^{2+} and PO_4^{3-} into the bloodstream. This process has been demonstrated to coordinate with fibroblast growth factor 23 (FGF23), which is released by the bone, and acts on multiple organs involved in Ca^{2+} homeostasis.[75] FGF23 has a particularly important role in regulating permeability of Ca^{2+} in the small intestines by negatively regulating the influence of $1,25(OH)_2D$ via increased production of 24-hydroxylase.[76]

CaSR influences a wide variety of different signaling processes depending on its expression location. For example, the proliferation of osteoblasts[77] and mucous epithelial cells in the stomach[78] is promoted by increased expression of CaSR. Alternatively, parathyroid cells[79] and intestinal cells in vitro[80] experience attenuated proliferation as a function of CaSR cell surface expression. Intestinal cell differentiation is also negatively regulated in vitro.[81] When CaSR activity is increased in osteoclasts, bone resorption is downregulated. Meanwhile, the proliferation and differentiation of osteoclasts were enhanced via PLC activation and subsequent nuclear translocation of pro-inflammatory transcription factors, such as the nuclear factor κB cells.[82,83] In the kidneys, CaSR recognition of $[Ca^{2+}]_o$ inhibits renin secretion by juxtaglomerular cells[6] and expression of aquaporin-2 in the collecting ducts of the kidneys.[84] The balance between these different cellular modalities (e.g., proliferation vs. senescence) is linked to the oncogenic potential of various cells. CaSR has been previously considered a tumor suppressor in colorectal cancers[85,86] and neuroblastomas,[87] but it conversely acts as an oncogene in breast cancer[88] and prostate cancer.[89]

CaSR mutants, or mutants of proteins that interact with CaSR, may contribute to the pathology in disorders of Ca^{2+} homeostasis and cause calciotropic diseases. This is frequently a consequence of aberrant and defective ligand binding and trafficking of the protein toward the plasma membrane.[90,91] High blood Ca^{2+} concentrations cause hypercalcemic diseases and may result from either one or two inactivating mutations in the CaSR gene. Conversely, low blood Ca^{2+} concentrations cause hypocalcemic diseases. Loss-of-function mutations may decrease the sensitivity of CaSR signaling beyond the equilibrium exhibited in the wild type, while gain-of-function mutations can increase the sensitivity of the CaSR below the equilibrium exhibited in the wild type. Examples of diseases caused by loss-of-function CaSR mutations include familial hypocalciuric hypercalcemia 1 (FHH1), neonatal severe hyperparathyroidism (NSHPT), and hyperparathyroidism, while gain-of-function diseases include autosomal dominant hypocalcemia 1 (ADH1) and hypoparathyroidism. Loss-of-function mutations either occur in a heterozygous form, causing FHH, or in a homozygous form, causing NSHPT.[92] Conversely, gain-of-function mutations give rise to hypocalcemic disorders such as ADH and Bartter syndrome type V.[56] Many therapeutics have been developed to treat these diseases, with several small-molecule treatments, such as cinacalcet and evocalcet, and biologics, such as etalcalcetide.

CaSR is also expressed in tissues including the central nervous system, cardiovascular system, lungs, gastrointestinal system, pancreatic islets, adipose tissue, skin, and other organs that are not involved in extracellular Ca^{2+} homeostasis.[56,93] Evidence suggests that CaSR also regulates the pathophysiology of noncalciotropic diseases. CaSR also plays a role in the differentiation of cerebellar granular cells, in the sympathetic portion of the autonomous nervous system, and in oligodendrocytes in the brain.[94–96] CaSR affects various diseases including Alzheimer's disease, ischemic brain injury, and epilepsy in the nervous system.[93,97] A dinucleotide repeat in the CASR

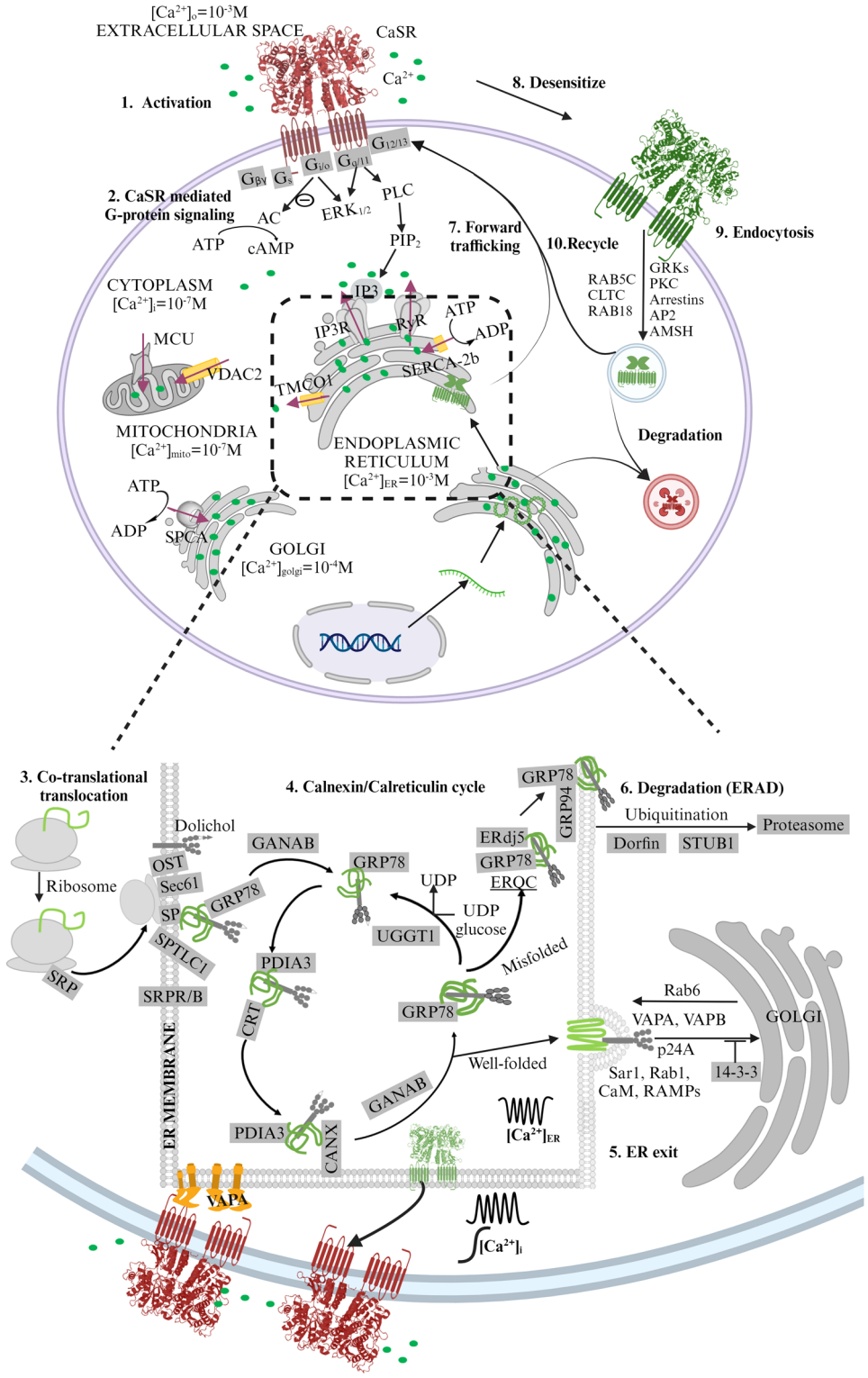

FIGURE 1 The Ca^{2+}-sensing receptor (CaSR) interactome characterizes the life cycle of CaSR and its signaling capacity. (1) Extracellular calcium activates CaSR and (2) mediates intracellular G-protein-coupled signaling, leading to endoplasmic reticulum (ER) calcium release by generating inositol 1, 4, 5-trisphosphate (IP_3) through phospholipase C (PLC) hydrolysis of PIP2. Treatment with extracellular calcium and alterations in ER calcium enrich the CaSR interactors, contributing to various processes within the ER (zoomed in). These processes include (3) cotranslational translocation, (4) quality control of the calnexin/calreticulin cycle, (5) ER exit of well-folded proteins, and (6) degradation of misfolded proteins. (7) The well-folded CaSR is trafficked to the cell membrane through agonist-driven insertion, where it can be (8) desensitized and (9) enter the cell through endocytosis. The endocytosed CaSR can either be (10) recycled back to the cell membrane or degraded in the lysosome.

gene has also been reported in one case of Alzheimer's disease.[98] Additionally, a decrease in CaSR heterodimers with $GABA_B1R$ is associated with ischemic brain injury in murine models.[99] Lastly, CaSR is involved in the regulation of blood vessel tone and, subsequently, endogenous blood pressure regulation.[100] CaSR promotes apoptosis in ventricular cardiomyocytes,[101] while it induces proliferation in vascular endothelial smooth muscle cells.[102] Notably, a CaSR SNP at residue A986S is associated with an increase in various cardiovascular diseases, including an increased risk of a heart attack.[103] There is also a strong link between CaSR and pulmonary hypertension, as well as asthma attacks associated with seasonal allergens. Following monocrotaline-induced pulmonary hypertension, CaSR is upregulated, which is associated with an increase in resting intracellular cytosolic Ca^{2+} and extracellular Ca^{2+}-induced cytosolic Ca^{2+} increase.[104] For more detailed information about CaSR in both calciotropic and noncalciotropic diseases, we recommend the excellent review previously published by Hannan et al.[93]

The importance of CaSR in calciotropic and noncalciotropic diseases highlights the importance of CaSR in translational medicine. Integrating the wide breadth of new information generated from recently published CryoEM structures will illuminate the molecular basis of CaSR-related diseases and inform the design of novel therapeutic agents that target CaSR and other family C GPCRs. We aim to first summarize recent advances in biochemical and structural determination of the CaSR and its interaction sites with Ca^{2+} and amino acids. We will also discuss CaSR regulatory mechanisms and its agonists and antagonists. We will then discuss CaSR disease mutations that are distributed around key ligand interaction and regulation sites to provide new insights into the molecular basis of acute and chronic CaSR-related diseases and the design of therapeutic agents that target CaSR and other family C GPCRs. Lastly, we will discuss the recent development of a Ca^{2+}-sensitive protein–protein interactome (PPI) for the calcium-sensing receptor (CaSR), which identified 94 novel proteins involved in CaSR-dependent protein folding, degradation, trafficking, cell surface expression, and signaling pathways involved in further CaSR-mediated signaling and functional cooperativity.

2 | STRUCTURAL AND REGULATION ADVANCES OF CASR AND THE LIGAND-BINDING SITES OF CASR

Huang et al.[105,106] from the Yang group reported in 2007 a modeled structure of the ECD of CaSR with the Venus fly trap (VFT) domain, based on 27% sequence identity between the CaSR ECD and the X-ray structures of mGluR1 (PDB ID: 1EWR and 1ISR). Further, by using computational algorithms that performed statistical analysis of coordination properties of known Ca^{2+}-binding proteins, five Ca^{2+}-binding sites were predicted in each protomer of the CaSR ECD: Site 1 (S147, S170, D190, Y218, and E297), Site 2 (D215, L242, S244, D248, and Q253), Site 3 (E224, E228, E229, E231, and E232), Site 4 (E350, E353, E354, N386, and S388), and Site 5 (E378, E379, T396, D398, and E399)[50,105,107] (Figure 2). The Ca^{2+}-binding capability and binding homocooperativity of the predicted Ca^{2+}-binding sites with one another were determined experimentally by either grafting them individually into a non-Ca^{2+}-binding protein CD2 (Sites 1, 3, and 5)[105] or by engineering subdomains encompassing several wild-type or mutated Ca^{2+}-binding sites using tryptophan-terbium fluorescence resonance energy transfer binding and Ca^{2+} competition binding assays, Trp fluorescence, and ^1H nuclear magnetic resonance (NMR).[50] An amino acid binding site adjacent to the calcium-binding Site 1 at the hinge region of the CaSR ECD was identified using structure modeling, molecular docking, and functional assays. Utilizing saturation transfer difference NMR and the purified CaSR ECD, Zhang et al.[108–110] determined the binding affinity of l-Phe to the CaSR ECD, and this binding was enhanced in the presence of Ca^{2+} with heterotropic cooperativity. This work led to a proposed working model for the co-activation of the CaSR via both Ca^{2+} and amino acids at the hinge region of the ECD.[108,109]

Later in 2016, Zhang et al.,[111] also from the Yang lab, reported the first structure determination of an Mg^{2+}-bound form of native CaSR ECD dimer (residues 1–540), with reduced glycosylation at pH 7.0, and at a resolution of 2.1 Å (PDB ID: 5FBK), as well as a Gd^{3+}-loaded form at 2.7 Å (PDB ID: 5FBK). Both determined crystal structures were very similar to their previously reported modeled structure.[111] Neighboring the Site 1 predicted by Zhang et al.,[111] an Mg^{2+}/Ca^{2+}-binding site at the hinge region (D216, D275, and S272), referred to as Site 1b, was revealed (Figure 2). At the region around the original predicted Site 3, a metal-binding site formed by negatively charged residues E228, E231, S240, and E241 (acidic patch) was identified at the homodimer interface of VFT Lobe 2 (referred to as Site 2). After soaking the crystal in the presence of Gd^{3+}, this site appeared to be coordinated by a different grouping of chelators (E228, E229, and E232). Additionally, a new metal-binding site (Site 3), formed by peptide backbone carbonyl oxygen atoms from uncharged residues (new site: I81, S84, L87, and L88) (Figure 2), was revealed. Predicted Sites 4 and 5 could not be verified, primarily due to the conformational ensemble related to their locations at the invisible loop in the determined structures.

Subsequently, Geng et al.[53] determined both apo- and holo- forms of the CaSR ECD with mutations in various glycosylation sites and in the Cys-rich domain under different conditions of pH and at pH 4.2 with different concentrations of Ca^{2+}, phosphate, and sulfate

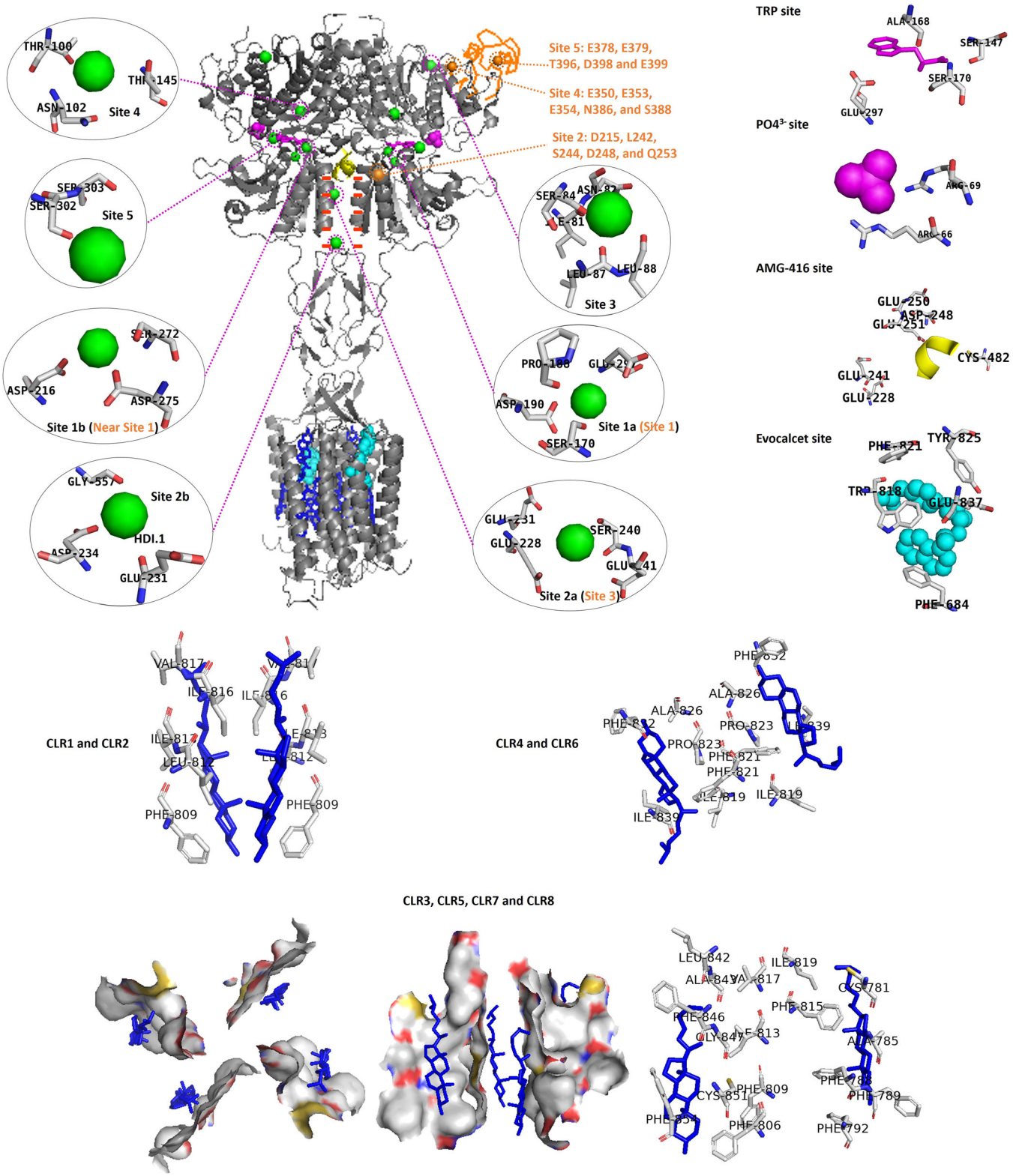

FIGURE 2 Modeled Ca^{2+}-sensing receptor (CaSR) structure with summary of all reported (black font) and predicted (orange font) interaction sites with Ca^{2+}, amino acids, and pharmacological agents. Green spheres represent Ca^{2+}. L-Trp is indicated by magenta sticks. PO_4^{3-} ions are shown in magenta spheres. AMG-416 is indicated as a yellow ribbon, evocalcet is shown in cyan spheres, and cholesterols are indicated as blue sticks. The acidic patch at the homodimer interface is shown as two sets of red dashes ("-"), parallel to one another.

(SO_4^{2-}) (PDB ID: 5K5S and 5K5T) with holo-ECD structure (PDB ID: 5K5S) at 2.6 Å resolution in 1.6 M Na_2HPO_4, 0.4 M K_2HPO_4, 100 mmol/L Na_2HPO_4/citric acid, 10 mmol/L $CaCl_2$, and 10 mmol/L L-Trp. Alternatively, the apo-ECD structure (PDB ID: 5K5T) was resolved at 3.1 Å in 1.5 M Li_2SO_4, 100 mmol/L Tris, and 2 mmol/L $CaCl_2$, at pH 8.5. The novel Site 3 identified by Zhang et al. from Yang lab was also later identified in the 5K5T structure, in addition to a metal-binding Site 2b at a slightly lower position compared to Zhang et al.[111] (Site 2a), coordinated by D234, E231, and G557, which was identified in the acidic patch of the dimer interface. However, Ca^{2+} binding Sites 4 (T100, N102, and T145) and 5 (S302 and S303) in the 5K5T structure, both of which lacked negatively charged ligand residues, were interpreted as chloride-ion binding sites in the 5FBK structure by Zhang et al.[111]

Unexpectedly, Zhang et al.[111] also identified a tryptophan derivative, L-1,2,3,4-tetrahydronorharman-3-carboxylic acid (TNCA), bound by S147, S170, D190, Y218, and E297, at the hinge region in the 5FBK crystal structure, based on X-ray density and mass spectroscopy. TNCA, also referred to as cyclotryptophan, was later reported at the same site by others in several determined structures using EM[112,113] (Figure 2 and Table 1). Conversely, Geng et al.[53] reported that the same region in the 5K5S structure was occupied by lL-Trp-Trp rather than TNCA (Figure 2). Structural modeling studies also suggested that γ-glutamyl peptides such as glutathione, oxidized glutathione, and oxidized glutathione derivative L-cysteine glutathione disulfide likely bind at the same hinge region of the CaSR ECD.[114–116] It is possible that CaSR may also have the capacity to distinguish between oxidizing and reducing cellular environments.[116]

The identification of potential anion binding sites by Zhang et al.[111] (e.g., HCO_3^-) and Geng et al.[53] (e.g., PO_4^{3-}) adjacent to Site 1 and the amino acid/TNCA at the hinge region suggests that anions likely function as negative modulators of CaSR to further mediate CaSR-dependent conformational changes induced by coactivation of Ca^{2+} and amino acids.[106] Extracellular association with arginine residues and intracellular covalent modifications of CaSR by phosphate have been suggested as mechanisms in which PO_4^{3-} influences conformational changes.[117] In sperm cells, CaSR has been hypothesized to function as a pH sensor in the acidic female reproductive tract by recognizing local HCO_3^- gradients and transducing these graded changes into guidance cue signals.[118] CaSR activation also influences secretion of HCO_3^- levels in the colon, suggesting that it may be able to act upon other CaSR receptors in a paracrine manner via its sensitivity to HCO_3^-. Notably, the release of HCO_3^- in the colon is dependent on short-chain fatty acids and Cl^- and is independent of local cAMP concentrations.[119,120] Thus, the summation of excitatory and inhibitory ligand binding to CaSR, especially in the VFT, encodes graded levels of local CaSR activation.

2.1 | Structures of CaSR using Cryo-electron microscopy (Cryo-EM)

Due to recent advances in Cryo-EM and structural determination, a total of 22 additional CaSR structures have been published[112,113,121–124] (Table 1). The "open" or "inactive" state conformational structures (PDB ID: 7M3J, 7SIN, 7E6U, 7DTU, 7DTW) include two that are in complex with antagonist NSP-2143 (PDB ID: 7M3J,[123] 7SIN[112]), one with artificial inhibitory nanobody NB-2D11 (PDB ID: 7E6U[113]), one with the only known co-agonist TRP (PDB ID: 7DTU[122]), and one in absence of any agonist ligand molecules (PDB ID: 7DTW[122]). The "activated" structures are observed in the presence of various endogenous allosteric ligands, such as TNCA (PDB ID: 7SIM[112]) and chloride (Cl^-) anions (PDB ID: 7DD6[121]), or in the presence of high concentrations of Ca^{2+} (PDB ID: 7M3F[123]), or in complex with exogenous modulators.

CaSR-published structures in complex with positive allosteric modulators (PAMs) include two with evocalcet (PDB ID: 7DD7,[121] 7M3G[123]), six with cinacalcet (PDB ID: 7M3F,[123] 8WPU,[125] 8WPG,[125] and 8SZ(F-H) with spermine[124]), and one with R-568 (PDB ID: 7SIL[112]). Cinacalcet and evocalcet are two calcimimetic agents that have been approved for the treatment of secondary hyperparathyroidism in dialysis patients with chronic kidney disease and hypercalcemia with parathyroid carcinoma in the United States[126] and Japan,[127] respectively. An additional structure also included 1 M of cinacalcet in their sample preparation, but cinacalcet did not appear in the published structure (PDB ID: 7E6T[113]). One of the active CaSR structures (PDB ID: 7M3G[123]) was published with both evocalcet and etalcalcetide (AMG416), a peptide made of D-amino acids that is administered after hemodialysis in patients with secondary hyperparathyroidism and chronic kidney disease.[128] Interestingly, etalcalcetide (AMG416) binds to the negatively charged acidic ligand-binding site (E251, C482, E228, E241, D248, E250) at the bi-lobed dimer interface, adjacent to the identified Ca^{2+}-binding Site 2a.[12] In a recent Cryo-EM structure of CaSR with cinacalcet embedded in lipid nanodiscs, spermine was shown to occupy the negatively charged dimeric site at Lobe 2 of the ECD, involving residues E228, E232, E238, and E241, and at the ECD-cysteine-rich domain (CRD) region, involving residues E604, E755, E757, and Y829.[124] In 2023, Ling et al.[125,129] reported two CaSR structures in complex with cinacalcet with (PDB ID: 8WPU) and without (PDB ID: 8WPG) heterotrimeric G_q proteins. In 2024, He et al.[124] reported three structures (PDB ID: 8SZI, 8SZH, 8SZG) of CaSR complexed with G_i and G_q proteins in the presence and absence of cinacalcet.

TABLE 1 Calcium-binding sites in CaSR ECD.

PDB ID	1st author	Journal	Method	Resolution	Ligands	CRD	TM	ICD	Calcium-binding sites	Paper DOI
5FBH	C. Zhang	*Sci Adv*	X-ray	2.7	BCT, CL, GD_3, MG, NAG, TNCA	X	X	X	Sites 1b, 2a, and 3	https://doi.org/10.1126/sciadv.1600241
5FBK	C. Zhang	*Sci Adv*	X-ray	2.1	BCT, CL, MG, NAG, TNCA	X	X	X	Site 3	
5K5S	Y. Geng	*Elife*	X-ray	2.6	CA, NAG, PO_4, TRP	□	X	X	Sites 2b, 3, 4, and 5	https://doi.org/10.7554/eLife.13662
5K5T	Y. Geng	*Elife*	X-ray	3.1	CA, NAG, SO_4	□	X	X	Site 4	
7DD5	T. Wen	*Sci Adv*	Cryo-EM	3.2	CA, CL, NAG, TRP, NSP-2143	□	□	X	Sites 2b, 4, and 5	https://doi.org/10.1126/sciadv.abg1483
7DD6	T. Wen	*Sci Adv*	Cryo-EM	3.2	CA, CL, NAG, TRP	□	□	X	Sites 2b, 4, and 5	
7DD7	T. Wen	*Sci Adv*	Cryo-EM	3.2	CA, CL, Evocalcet, NAG, TRP	□	□	X	Sites 4 and 5	
7DTT	S. Ling	*Cell Res*	Cryo-EM	3.8	CA, NAG	□	□	X	Sites 2b and 5	https://doi.org/10.1038/s41422-021-00474-0
7DTU	S. Ling	*Cell Res*	Cryo-EM	4.4	NAG, TRP	□	□	X	N/A	
7DTV	S. Ling	*Cell Res*	Cryo-EM	3.5	CA, NAG, TRP	□	□	X	Sites 2b and 5	
7DTW	S. Ling	*Cell Res*	Cryo-EM	4.5	NAG	□	□	X	N/A	
7E6T	X. Chen	*Elife*	Cryo-EM	3.0	CA, NAG, PO_4, TNCA	□	□	X	Sites 1a, 2b, and 4	https://doi.org/10.7554/eLife.68578
7E6U	X. Chen	*Elife*	Cryo-EM	6.0	NB-2D11 (protein)	□	□	X	N/A	
7M3E	Y. Gao	*Nature*	Cryo-EM	3.2	CA, NAG, PO_4, TRP, NSP-2143	□	□	X	Sites 2b and 3	https://doi.org/10.1038/s41586-021-03691-0
7M3F	Y. Gao	*Nature*	Cryo-EM	2.8	CA, NAG, PO_4, TRP, Cinacalcet	□	□	X	Sites 2b and 3	
7M3G	Y. Gao	*Nature*	Cryo-EM	2.5	CA, Evocalcet, NAG, PO_4, TRP, AMG-416	□	□	X	Sites 2b and 3	
7M3J	Y. Gao	*Nature*	Cryo-EM	4.1	NAG, PO_4, NSP-2143	□	□	X	N/A	
7SIL	J. Park	*PNAS*	Cryo-EM	2.7	9IG, CA, CLR, NAG, PO_4, TNCA	□	□	X	Sites 2b, 3, 4, and 5	https://doi.org/10.1073/pnas.2115849118
7SIM	J. Park	*PNAS*	Cryo-EM	2.7	CA, CLR, NAG, PO_4, TNCA	□	□	X	Sites 2b, 3, 4, and 5	
7SIN	J. Park	*PNAS*	Cryo-EM	5.9	NSP-2143	□	□	X	N/A	
8WPU	S. Ling	*Cell Res*	Cryo-EM	3.1	CA, CLR, PO_4, TRP, Cinacalcet, NAG, G_q ($\beta\gamma$)	□	□	X	Sites 2b and 3	https://doi.org/10.1038/s41422-023-00892-2
8WPG	S. Ling	*Cell Res*	Cryo-EM	2.7	CA, PO_4, TRP, Cinacalcet, NAG	□	□	X	Sites 2b and 3	
8SZI	F. He	*Nature*	Cryo-EM	3.5	CA, PO_4, TRP, NAG, CLR, PCW, G_i ($\beta\gamma$)	□	□	X	Sites 2b and 3	https://doi.org/10.1038/s41586-024-07055-2
8SZH	F. He	*Nature*	Cryo-EM	3.1	CA, PO4, TRP, SPM, Cinacalcet, NAG, CLR, PCW, G_i ($\beta\gamma$)	□	□	X	Sites 2b and 3	
8SZG	F. He	*Nature*	Cryo-EM	3.6	CA, PO_4, TRP, SPM, Cinacalcet, NAG, CLR, PCW, Gq ($\beta\gamma$)	□	□	X	Sites 2b and 3	

TABLE 1 (Continued)

PDB ID	1st author	Journal	Method	Resolution	Ligands	CRD	TM	ICD	Calcium-binding sites	Paper DOI
8SZF	F. He	*Nature*	Cryo-EM	2.8	CA, PO_4, TRP, SPM, Cinacalcet, NAG	□	□	X	Sites 2b and 3	

Note: Site 1a (S147, S170, D190, Y218, and E297), Site 1b (D216, D275, and S272) next to Site 1a, Site 2a (E224, E228, E229, E231, and E232), Site 2b (D234, E231, and G557) lower than Site 2a, Site 3 (I81, S84, L87, and L88), Site 4 (T100, N102, and T145), Site 5 (S303 and S302).

Abbreviations: 9IG, 3-(2-chlorophenyl)-N-[(1 R)–1-(3-methoxyphenyl)ethyl]propan-1-amine (R-568); BCT, bicarbonate ion; CA, calcium; CL, chloride ion; CLR, cholesterol; CRD, cysteine-rich domain; CryoEM, Cryo-electron microscopy; ECD, extracellular domain; GD3, gadolinium ion; ICD, intracellular domain; MG, magnesium ion; NAG, 2-acetamido-2-deoxy-β-D-glucopyranose; PCW, 1,2-dioleoyl-sn-glycero-3-phosphocholine; PO_4, phosphate ion; SO_4, sulfate ion; SPM, spermine; TRP, tryptophan; TNCA, 1,2,3,4-tetrahydronorharman-3-carboxylic acid.

Unexpectedly, in addition to the hinge of VFT of ECD, L-Trp also occupied the same location of other PAM/negative allosteric modulator (NAM) at the 7-transmembrane domain (7TMD) but was shown to have no allosteric effects after truncating the CaSR ECD.

These recent cryo-EM structures further validated the Ca^{2+}-binding sites reported and proposed by Zhang et al. and Geng et al.[53,111–113,121–123] The structures from Ling et al.,[122] Geng et al.,[53] and Wen et al.[121] identify the same Ca^{2+}-binding site in the region between VFT and the CRD. In addition, several binding sites for known or possible CaSR modulators were identified, including a common site (R66, R69) for several anions, a negatively charged acidic ligand-binding site for etalcalcetide (AMG416), and several cholesterol sites in 7TM near the TM6-TM6 dimer interface[12] (Table 1 and Figure 2). CaSR has been observed in bovine parathyroid cells to localize to cholesterol and caveolin-rich segments of the plasma membrane, which suggests an important physiological role for cholesterol and cholesterol-dependent activation of CaSR.[112,123,125,130] Residues F809, L812, and I813 have been implicated in complex formation with molecules of cholesterol. F809L is an inactivating mutation of CaSR that is implicated in pathogenesis of FHH1.[131] Mutating the cholesterol-binding residues to alanine resulted in decreased basal activity of CaSR[112] (Figure 2). There is also a putative cholesterol motif in this region, known as a CARC motif, between K805 and L812.[132]

2.2 | Key structural determinants in regulation of CaSR

Increasing evidence supports a working model wherein increases in extracellular Ca^{2+} are required to activate CaSR by working with endogenous PAMs (i.e. either amino acids or cyclotryptophan/TNCA) at the hinge region (Site 1) of the ECD and the highly negatively charged dimerization site (Site 2) between two CaSR protomers (Figure 3B).[105,111] This is also observed with other closely related class C GPCRs, such as mGluRs, which sense the amino acid glutamate and $[Ca^{2+}]_o$ via binding pockets in the ECD,[133] and in the $GABA_BRs$, which sense an important glutamic acid decarboxylase (GAD)-dependent metabolite of glutamate, known as γ-aminobutyric acid, or GABA.[134] Upon binding of Ca^{2+} and amino acid/TNCA to the hinge region, Ca^{2+} then binds to the acidic homodimer interface of VFT Lobe 2, inducing conformational changes at CRD and the TMD with movement of TM6 and possibly the C-terminal tail. Anions, on the other hand, have been demonstrated to serve as endogenous NAMs, with SO_4^{2-} ions producing an increase in EC_{50}.[53] Each protomer of CaSR participates in a network of molecular interactions, including hydrogen bonding between the linker region (C-terminal to the CRD) amino acid residues and the

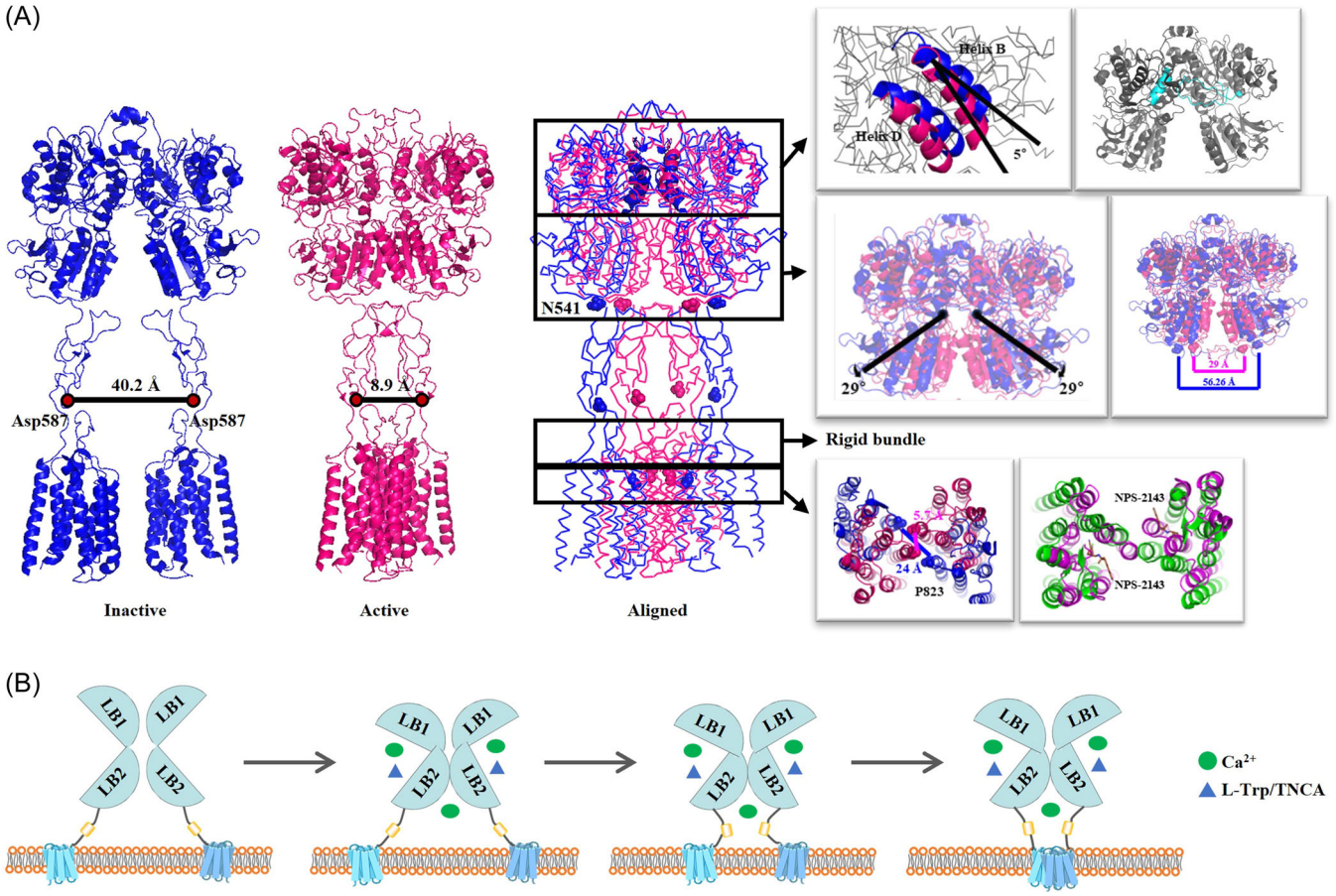

FIGURE 3 Working model of Ca^{2+}-sensing receptor (CaSR) activation. (A) Comparison of structural conformation changes between activated and inactivated CaSR. The inactivated (PDB ID: 7DTW) and activated (PDB ID: 7DTV) CaSR structures are shown as blue and magenta cartoons, respectively, and the aligned structures are presented as ribbons. Asp587 is highlighted as a red spot in the cartoon structure and as a sphere in the aligned structure. The helix and extended loop connecting the two LB1 subunits is highlighted in cyan. The rigid structure bundle linking cysteine-rich domain (CRD) and transmembrane domain (TMD) is highlighted and compared with magenta cartoon (left) and amino acid sticks (right) in the third frame. The TMD from the intermediate structure (PDB ID: 7DD5) is color-coded in green and aligned with the fully activated structure. This alignment implies that NSP-2143 is lodged in the intermediate TMD, impeding the rotation process. (B) Working model of the conformation change during CaSR activation by Ca^{2+} and amino acid/1,2,3,4-tetrahydronorharman-3-carboxylic acid (TNCA). From left to right, inactive CaSR, CaSR with closed extracellular domain (ECD), CaSR with closed ECD and closer aligned Cys-rich domain, fully activated CaSR. The green sphere represents calcium ions, and blue triangle represents L-Trp or TNCA.

extracellular loops (ECLs) of the 7TMD and π^+ stacking interactions between W590, K601, and I761.[112]

The transition of CaSR from its relaxed and open inactive state conformation to the compact activated conformation involves intricate conformational alterations across various structural domains and multiple intermediate substates. As seen in Figure 3, in the inactive structure, the VFT, CRD, and 7TMD are distinctly isolated, exhibiting obvious interdomain gaps (blue cartoon). Conversely, the active CaSR conformation (magenta cartoon) is more compact, with a solvent-accessible surface area of 3378 Å buried within the structure. In contrast, the inactive CaSR structure conceals a significantly smaller 1346 Å solvent-accessible surface area.[113]

The ECD VFT domains of all activated structures share strong structure homology with the first reported structure by Zhang et al.[111] (PDB ID: 5FBK). When superimposing the structures of Geng et al. (PDB: 5K5S) and Ling et al. (PDB: 7DTV) with Zhang et al. (PDB: 5FBK), a precise alignment is observed in the tightly packed VFT region, with a root-mean-square deviation (RMSD) of 0.64 Å (calculated using PyMOL).[53,121,122,135] The coordinated presence of Ca^{2+} and L-Trp/TNCA, which induces the VFT movement, exhibited near-perfect overlap at the LB1-LB2 cleft. Additionally, the structure from Wen et al., featuring an active ECD but inactive TMD, also demonstrated a significant resemblance to the work by Zhang and Ling in the VFT region, with an RMSD of 0.55 Å.[121]

Binding of both Ca^{2+} and Trp/TNCA at the hinge region (Site 1) and Ca^{2+} binding at the highly negatively charged dimerization surface (Site 2) involves critical conformational changes to establish hydrogen bonds

and hydrophobic interactions in these regions.[111,123] Binding of Ca^{2+} at Site 1 and adjacent AA/TNCA binding site through interactions with D190, E297, P188, S170, and Y489 induce the closure of the VFT.[113] This involves inward rotation and upward movement of LB2 and 5° rotation of LB1, which has a loop (F38–N64) spanning the homodimer interface. This engages hydrophobic interactions with the LB1 helix of another subunit (E456–H466) and F444 (Figure 3), as first observed in Zhang et al.[112,113,122] In the absence of Ca^{2+} binding during the inactive state, repulsive interaction between negatively charged residues (the previously noted acidic patch) at Site 2 leads to a separation of 56.26 Å between the C-termini of the two LB2 domains, based on the relative position of N541. Ca^{2+} binding at Site 2 reduces the distance of LB2 to ~40 Å at an intermediate state and is further reduced to 29 Å in the fully active state (Figure 3).[113] Ca^{2+} binding also results in a 29° inward rotation of LB2 with respect to LB1.[53,113] The close proximity and twisting of the two LB2 domains results in the formation of a new interaction interface between the homodimers, leading to the signature transition of the VFT from an open to a closed and fully activated conformation of CaSR.

In contrast, NB-2D11 binding at the cleft between two LB2 dimers prevents conformational motion that would reduce the distance between the LB2 homodimers and is characteristic of the inactive CaSR structure.[113] Mutations at key residues, such as W70, T145, A168, and S170, all located at the hinge region, largely diminish the associated activation process and reduce functional cooperativity.[53,111,115,122,136,137] Additionally, mutation E228I located at the dimeric groove dramatically reduces cooperative Ca^{2+} responses orchestrated by TNCA, which further suggests a dual action between Ca^{2+} and amino acids (or TNCA), via heterotropic cooperativity.[106,111] Stabilization of Ca^{2+} binding to the negatively charged patch at Site 2 is supported by a disulfide bond formed between C236 and C561, which relays the induced conformational change from the VFT domain to the TMD to induce close contact between the two CRD and two TMDs of the dimeric receptor in the fully active state.[53] As the VFT undergoes distortion, the distance between Asp587 within CRD is reduced from 40.2 Å to 8.9 Å during the transition from intermediate to active states, leading to closer alignment of the two CRDs. Meanwhile, the distance between their C-termini contracts from 83 Å to 23 Å upon CaSR activation.[53,122] Thus, Ca^{2+} binding facilitates distortion of the VFT, which is subsequently transmitted to the TMD through the CRD.[112,122] In both the inactive and intermediate state structures of CaSR, the two TMD subunits are entirely separated, with a plane distance of 24 Å.[113] Ca^{2+} binding at the ECD region also supports formation of a TMD homodimer, which presents new interaction interfaces resulting from reorientation of TM5/TM6. Notably, the highly positively charged agonist AMG416 interacts at the negative charged dimeric surface to stabilize activated conformation of the CaSR.[123]

The second extracellular loop (ECL2) of CaSR plays an important role in signaling and facilitating the conformational transition from CRD to TMD,[138] similar to the mGluR proteins, with high homology. The elongated peptide sequence (L601–E604) at the CRD C-terminus and the ECL2 (E759–I763) establish a rigid anchoring site for TMD movement with hydrophobic interactions (Figure 3).[113,122] In addition, two cysteine residues located on ECL2 and TM3 (C765 and C677, respectively) form a disulfide bond, contributing significantly to the conformational transition.[122] The reorientation process is significantly influenced by interactions at specific residues, including E759, Y590, and K601.[112,113,122] In the quiescent state of the CaSR, TM5-TM6/TM5-TM6 constitutes the closest plane within the TMD homodimer interface with the closest proximity (17 Å) observed between the two TM6 helices.[52,113] Following the conformational transition, the TMD is converted into the active phase, primarily governed by the robust association at the TM6–TM6 interface. Closer proximity at this interface is facilitated by an α-helix break that occurs between F821 and P823 of the TM6.[123] Notably, the S827 residues, positioned at the N-terminus of each TM6, are located within 5.7 Å of each other (Figure 3).[122] In the final stage of the reorientation of the TMD, which brings the 7TMs in closest proximity with a TM6-TM6 interface, CaSR completes the transition from an open, inactive conformation, to a condensed, active state.

A recent study conducted by Ling et al.[122] reported that the G_q α5 helix interacts with CaSR through polar interactions involving residues R701, E707, and K709, in ICL2. It also interacts through hydrophobic interactions, specifically between residues V702, V705, and F706, in ICL2, and F801 in ICL3, which is also conjugated to TM6. The interaction of the G_q protein further causes conformational rearrangements of the 7TM, with ICL2 extending to the cytosolic space and ICL and ICL3 moving toward the center of the 7TM bundle. In the nanodisc structures published by He et al.,[124] both G_i and G_q introduced additional conformational changes in the activated CaSR dimer complexed with cinacalcet and lipid interactions. The differential coupling of G proteins is involved in the rearrangement of ICL2 and the C-terminus of CaSR.[124] These data strongly suggest that the rearrangement of 7TM is pivotal for G_q and G_i protein interaction. Furthermore, the formation of the 7TMD homodimer during CaSR activation seems to provide the necessary environment conducive to these crucial interactions.[125,139]

Due to the intrinsically disordered structure of the CaSR C-terminal domain, all reported CaSR structures have been unsuccessful in defining any secondary structure in the region from sequence residue 890 to

the C-terminal domain (i.e., C-terminal tail). This region largely contributes to trafficking and regulation of the receptor at the plasma membrane and network pathways that will be summarized in Section 4.[140]

3 | MOLECULAR BASIS OF DISEASE MUTATIONS IN CASR

3.1 | CaSR mutations in the ECD

Recent advances in structure and key regulation determinants also provide molecular insights for the consequences of disease-related mutations. More than 400 mutations in the CaSR have been identified causing various disorders. Among these mutations, over 225 are specifically mapped to the ECD, comprising >56% of the known disease-causing mutations related to CaSR.[111] Mapping of disease-associated mutations on the structure of human CaSR-ECD demonstrates that the mutations are clustered in two regions with pivotal roles in CaSR function: the hinge region between Subdomains 1 and 2 (e.g., E297K) and the dimerization interface between two Lobe 2 segments that are important for Ca^{2+} and ligand binding (Figures 3 and 4).[56,110] ADH disease patients have been identified with mutations in the known Ca^{2+} binding sites in the lobar interface of the VFT domain. There have been three observed, distinct missense mutations at E228 (E228K, E228G, and E228Q) and one mutation at E297 (E297D).[110,141–144] Reduced CaSR membrane expression has been associated with several identified disease mutations in the ECD, including mutations Y218S, Y218C, and E297K in FHH, and E228Q and E241K in ADH.[145,146] It is noteworthy that residues P221 and E297 have the capacity to result in either CaSR loss of function or gain of function when mutated. Specifically, the E297K and P221Q mutations are associated with FHH, while the E297D and P221L mutations are linked to ADH.[147–149]

Loss-of-function mutations, such as P55L, C60F, R66C and R66H, are clustered near binding sites for anions, such as PO_4^{3-} and HCO_3^- in neighboring arginine residues R66 and R69.[53,110,150–157] Disease mutations impact the ability for protomers (i.e., monomers) of CaSR to form obligate dimers, such as C482Y, which also leads to aberrant receptor function.[114,158] Cysteine disulfide bonds and hydrophobic interactions influence the formation of CaSR dimers.[159] Although the CRD of a CaSR monomer primarily forms cysteine-cysteine disulfide bonds to influence the tertiary structure of the monomer, disulfide bridges are also formed between CaSR protomers at C129 and C131.[160] It has been reported that several missense mutations at these two cysteine residues (C129Y, C129F. C129S, C131Y, C131F, and C131W) can also cause ADH.[144]

Other ECD mutations include heterozygous mutants in G143E and R185Q, which frequently cause FHH-1.[161] Novel variants, both in exon coding regions and noncoding intron regions, are frequently discovered. For example, a heterozygous mutation at L606P was identified in a patient with NSHPT, in addition to two rare intronic variants at chr3.g122180312A>G and chr3.g12225601G>A.[162] Mutant E586K was identified in a patient with ADH1 for the first time in 2023.[163] Notably, in 2023, two research groups—one in Israel[164] and one in China[165]—independently discovered an ADH-causing novel mutation at I139T in the VFT domain. These disease-associated mutations were found to alter the CaSR's responses to $[Ca^{2+}]_o$ and functional cooperativity between mutant heterodimers or mutant homodimers.[109] This recapitulates the importance of the integration of Ca^{2+} signaling from changes in $[Ca^{2+}]_o$ to intracellular signaling networks is critically important for many (patho)physiological processes.

3.2 | CaSR mutations in the TMD

More disease-related mutations are located within the 7TMD (Figure 4). Gain-of-function mutations are primarily but not exclusively localized in the TMD of the full-length human CaSR, which contrasts with the full-length human CaSR loss-of-function mutations, which are approximately distributed normally across all domains of the protein.[121] Two cases of ADH1 disease mutations in human patients were reported within the TMD6 region, specifically, at residues W818L, Y825P, and E837D.[110,166–168] A gain-of-function mutation was also discovered at I823N in an ADH1 patient.[163] In addition, there are three separate published reports of a loss-of-function severe sporadic hypoparathyroidism mutation in human patients that expresses the exact same mutation transition, specifically at residue F821L.[168–170] Interestingly, the F821 residue in the 7TM region of CaSR has been identified as an important residue in the binding pocket of cholesterol in CryoEM structures of human CaSR, as well as the NAM, NPS-2143.[112] This residue F821 is also a focal point for the α-helix break on TM6 that induces the activation of CaSR in both the presence and absence of PAMs.[112]

3.3 | CaSR mutations in the intracellular domain (ICD)

Additionally, several disease mutations were identified in the unstructured cytosolic tail of the CaSR ICD largely involved in the alteration of CaSR's trafficking, surface expression, life cycle, and integration of signaling (Figure 4, see Section 4). The ICD of CaSR includes three PKC phosphorylation sites at T888, S895, and S915.[171] Phosphorylation at these sites is critical to

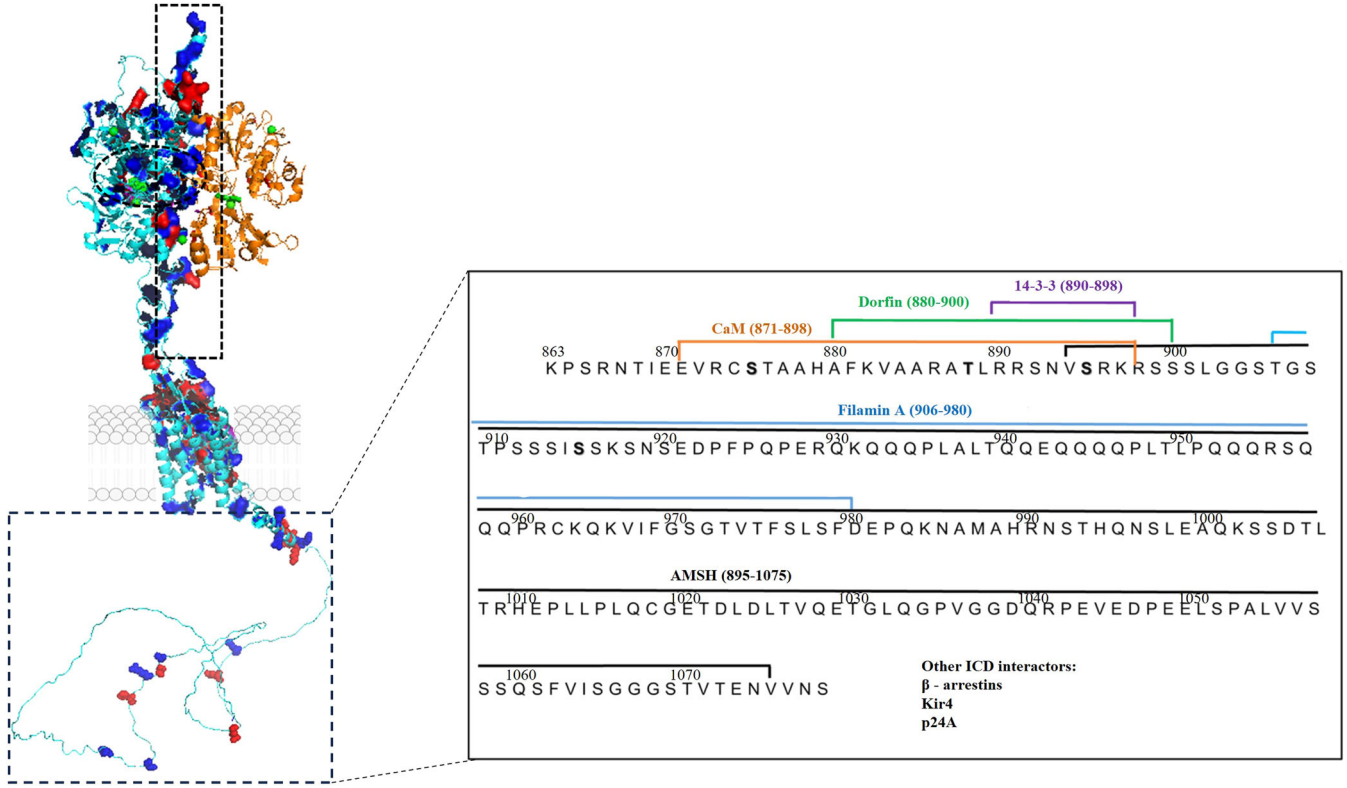

FIGURE 4 Disease-related mutations on Ca^{2+}-sensing receptor (CaSR). The full-length structure of CaSR, predicted by AlphaFold, was utilized to map disease mutations. (Top left) The CaSR hinge region is highlighted in a dashed circle, and the dimer interface is outlined within a dashed box. Color coding of amino acids in structure: residues reported with loss-of-function mutations (blue); residues reported with gain-of-function mutations (red); residues reported with both loss-of-function mutations and gain-of-function mutations (magenta). (Bottom left) Enhanced view of the CaSR intracellular domain (ICD) (863–1078) amino acid sequence that includes the CaSR interactors binding site. (Right) Phosphorylation sites (S875, T888, S895, and S915) are highlighted in bold in the sequence. The calmodulin binding site is marked orange, the Dorfin-binding site is marked green, the 14-3-3 binding site is marked purple, the filamin A binding site is marked blue, and the associated molecule with the SH3 domain of STAM (AMSH)-binding site is marked black. Other ICD interactors with unknown binding sites are listed beneath the sequence.

negatively regulate CASR downstream signaling.[71] It has been suggested that the CaSR missense mutation T888M at the phosphorylation site and deletion(s) between S895–V1075 cause gain-of-function disease ADH.[144,172] In addition, several loss-of-function mutations (R896H, A986S, and D1005N) were also reported in the ICD of CaSR.[144] Huang et al.[173] have shown that CaM binding to region 871–898 largely stabilizes CaSR's surface expression, which is important for functional cooperativity and calcium oscillations. A previously identified missense mutation Q926R in the CaSR C-terminal domain[174] was identified a second time in 2023 in a patient with both FHH and a single-gland parathyroid adenoma, ultimately causing the patient to clinically present with primary hyperparathyroidism. Interestingly, this residue is in a proline, glutamine, serine, and threonine (PEST) region in the C-terminal tail, associated with regulating cell surface expression and lysosomal degradation of CaSR, previously determined by truncation studies.[175] Another very recently discovered novel CaSR mutation was also identified in the C-terminal domain at T972M using induced human pluripotent stem cells from a patient with a heterozygous loss-of-function FHH mutation. It is possible that T972 could be a potential target of various kinases and phosphatases important for CaSR function. These discoveries reemphasize the important need to further elucidate the structure and function of the C-terminal tail and its potential role in regulation of CaSR's life cycle and signaling integration.

3.4 | Effect of CaSR mutations in the heterodimers for signaling

Lastly, CaSR is able to dimerize with other types of GPCRs, both in its wild-type and mutant forms. It may exist as a mutant heterodimer combining a mutant CASR protomer with a wild-type CASR protomer or as a subunit (mutant or wild-type) with other class C GPCRs, such as the T1R3 taste receptor,[176] mGluR1a,[177] $GABA_RB1$, or $GABA_RB2$.[178] One research group used heterodimer chimeras created using $GABA_RB1$ and $GABA_RB2$, with CaSR, to determine the effects of heterozygous gene mutations compared to homozygous wild-type and homozygous mutants.[179] In known

functional mutants of the VFT domain, only one wild-type VFT was necessary for the activation of Ca^{2+}_{ex}-induced CaSR signaling, with two VFT wild-type homodimers leading to a peak response. Alternatively, both functional wild-type TM6 domains are necessary for Ca^{2+}_{ex}-induced CaSR signaling. The same study also determined that signaling between subunits also occurs. The VFT of one monomeric CaSR was capable of transducing the conformational change necessary for TM6-dependent G-protein docking of the other monomer in the dimer.[179] Functionally, homodimers of CaSR are necessary to mediate the resting membrane potential of the cell through interactions with other ion channels in an inhibitory fashion, such as with Kir4.1/Kir4.2,[180] or in an activating fashion, such as with TRPC isoforms 3, 6, and 7.[181,182] The function of these channels may be influenced by heterozygous mutations that lead to some CaSR heterodimerization. Importantly, some CaSR homodimerization would still occur in an organism with a mutation, especially if the CaSR mutation is heterozygous or if it is a somatic mutation.

4 | INTERACTING PROTEINS AND REGULATORY NETWORK IN THE LIFE CYCLE AND SIGNALING OF CASR

4.1 | The first CaSR PPI illuminates the extensive regulatory network of CaSR

The synthesis of CaSR is followed by engagement with quality control complexes, which identify inaccurately folded receptors and direct them to the proteasome for degradation. Properly folded receptors will traffic to the plasma membrane or other cellular compartments, facilitated by interactions with chaperones and small GTP-binding proteins.[183] In a groundbreaking study by Gorkhali et al.[184] in 2021, the first CaSR-PPI network was characterized using quantitative proteomics with liquid chromatography tandem mass spectrometry (LC–MS/MS) coupled with co-immunoprecipitation (Co-IP), with and without extracellular Ca^{2+}, in HEK293 cells. This study uncovered 94 novel putative CaSR interactors alongside eight previously identified interactors, including $G\alpha_i$ and $G_{\beta 2}$. The novel CaSR interactors are encountered across the entire CaSR life cycle, facilitated by agonist-derived insertional signaling, from synthesis to insertion and including internalization and endocytosis processes.[184]

The life cycle of CaSR commences with the transcription and translation of the CaSR gene, followed by synthesis, folding, and postmodification within the ER–Golgi complex.[183,185] The matured CaSR is transported to the cell membrane, where it operates as a dimer, actively sensing alterations of extracellular Ca^{2+} and other endogenous ligands in the extracellular environment to trigger downstream signaling via interactions with heterotrimeric G proteins. The forward tracking of CaSR from the ER to cell surface is also controlled by agonists including extracellular Ca^{2+} and amino acids.[186] CaSR will then undergo desensitization and subsequent endocytosis. Once internalized, the fate of CaSR involves either recycling to the cell membrane or undergoing degradation within the proteosome and/or lysosome (Figure 1).[187,188] This intricate life cycle of the CaSR is regulated by numerous checkpoints and controlling factors, and the expression and functionality of CaSR are tightly regulated during each step of its life cycle.

4.2 | The CaSR gene

Human CASR maps on chromosome 3 q13.3-21.1 in a ~100 Kb locus, and the final processed transcripts include seven exons. The first exon is untranslated while exons 2–7 encode the CaSR protein of 1068 amino acids. Exons 1A and 1B encode for two alternative 5′-untranslated regions that may splice to a common segment encoded by exon 2. The transcription of CaSR is driven by either promoter P1 initiated with 1A or promoter P2 initiated with 1B.[189,190] It has been reported that exon 1A transcripts driven by P1 promoter are reduced in parathyroid tumors and colon carcinomas, while other investigations have suggested a concurrent decrease in both CaSR messenger RNA (mRNA) and protein expression specifically within parathyroid adenomas.[191,192] Simultaneously, examinations of the P2 promoter—a region characterized by its GC-rich composition—have revealed higher degrees of methylation in colorectal tumors compared to adjacent mucosal tissues. This increased methylation within the P2 promoter region may be associated with CaSR gene silencing and reduction of CaSR expression.[192,193] Studies have also suggested that vitamin D (1,25(OH) 2D3) regulates CaSR transcription, as CaSR mRNA was reduced in vitamin D-depleted rats. Notably, the injection of vitamin D upregulated CaSR mRNA levels in a dose- and time-dependent manner.[194,195] Lastly, pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6, all upregulate CaSR genes, likely via various transcription promoters.[192]

4.3 | CaSR interactors in protein folding and trafficking

Within the interactome, Gorkhali et al.[184] identified several proteins that may contribute to the co-translational translocation of CaSR in the ER immediately after its translation. The signal recognition particle (SRP), the SRP receptor (SRPR), and the protein transport protein Sec. 61 were detected, suggesting their

involvement in the initial translocation stage.[184] SRP recognizes the signal sequence of a newly synthesized peptide as it emerges from the ribosome, and studies have reported its function in directing the nascent protein to ER via interaction with SRPR on the ER.[196] Meanwhile, the Sec. 61 complex, a protein-conducting channel, facilitates the translocation of hydrophilic segments of client proteins across the ER membrane and integrates hydrophobic transmembrane segments into the ER membrane.[197]

The oligosaccharyl transferase (OST) complex subunits STT3B, RPN1, RPN2, and DDOST were present in the CaSR interactome, as were the glucosidase IIα subunit GANAB and the UDP-glucose glycoprotein glucosyltransferase 1 (UGGT1).[184] These subunits may mediate the glycosylation of CaSR and play an important role in protein quality control. The OST complex is well studied with respect to N-linked glycosylation. It transfers a tetradecasaccharide from a dolichol pyrophosphate donor to specific asparagine sides of nascent polypeptides in the lumen of the ER. Following this transfer, the ER modifies the tetradecasaccharide-containing proteins. Glucosidases such as GANAB then remove the outermost glucose residues and glucosyltransferases such as UGGT1 add diverse sugar residues where needed, contributing to the formation of intricate and diverse protein structures.[198,199]

The glycosylation and folding of nascent proteins occur concurrently within the ER and are tightly regulated by this protein quality control mechanism. The presence of CNX and CRT in the interactome suggests an involvement of the CNX/CRT cycle, which regulates glycoprotein-dependent protein folding. In addition, glucose-regulated protein 78 (GRP78) and protein disulfide isomerases PDI A3 and A6 were also identified by Gorkhali et al.[184] Although PDI regulates disulfide bond formation in nascent proteins, GRP78 together with CNX and CRT functions as chaperones to facilitate protein folding.[184,200,201] Within the CNX/CRT cycle, proteins will be examined at the check point after each round of deglycosylation. UGGT1 serves as a quality control regulator at this checkpoint.[202,203] Correctly folded proteins proceed to the Golgi network and improperly folded ones undergo ER-associated degradation. Otherwise, unfolded or intermediate proteins are subject to another round of interaction with CNX/CRT.

Gorkhali et al.[184] also reported that cargo receptor family member p24A (transmembrane emp24 domain) was more than fivefold enriched in the presence of high extracellular Ca^{2+} compared to EGTA in their interactome. Exocytosis of CaSR from the ER likely requires p24A, as it interacts with both cargo and COPI or COPII coatomers in the early secretory pathway. p24A interacts with the immaturely glycosylated form of CaSR at its C terminus and thereby increases CaSR stability in the ER. This promotes increased plasma membrane targeting of CaSR.[204,205] The Gorkhali study also reported the presence of protein 14-3-3, which binds at the arginine-rich domain of CaSR(R_{890}RxxxxRKR_{898}). This protein may play a role in ER retention of CaSR.[184,206–208] Receptor-activity-modifying proteins (RAMPs) may be also required for the forward trafficking of CaSR from the ER to the Golgi apparatus.[185,209]

CaM, also identified in the interactome, maintains CaSR surface expression and regulates CaSR-mediated signaling.[184] CaM likely binds residues 871–898 in the CaSR ICD. Disruption of the CaM-binding site may lead to significantly increased internalization of CaSR, which reduces functional cooperativity.[173,184] Membrane protein caveolins (Cavs) have been observed to localize with CaSR in several cell lines, including parathyroid cells and human osteosarcoma (Saos-2) cells, where CaSR intracellular loops ICL1 and ICL3 are likely the binding motifs.[130,210] Although no direct evidence has yet been found to suggest that caveolin contributes to CaSR plasma membrane localization, CaSR-associated signaling molecules including heterotrimeric GTP-binding proteins and protein kinase C family members are highly enriched in caveolae via the Cavs. For example, Cav-1 upregulates $[Ca^{2+}]_o$-stimulated $[Ca^{2+}]_i$ incrementation by CaSR.[210–212] Additionally, calpain 2 (m-calpain), a heterodimeric calcium-dependent cysteine protease, also colocalizes with CaSR in caveolae, participating in the degradation of membrane CaSR. Calpain inhibitors attenuate the release of lower molecular weight fragments of CaSR into the cell culture medium.[213]

CaSR is dynamically maintained in the membrane through a combination of agonist-driven insertion and desensitization actions. CaSR endocytosis often begins with PKC-mediated phosphorylation at the carboxyl terminal sites at residues T888, S895, and S915. Phosphorylation also occurs here by a family of serine/threonine protein kinases, known as G-protein-coupled receptor kinases (GRKs). Both PKC and GRKs have been identified in the interactome.[184,214–216] β-arrestins normally facilitate the process for uncoupling CaSR and G proteins before endocytosis.[217–219] Adaptor protein-2 has been shown to reduce CaSR endocytosis and is present in the CaSR interactome.[220] Ubiquitination of GPCRs at the plasma membrane can lead to endocytosis of receptors.[183,221,222] A low-molecular-weight CaSR construct was previously pulled down in a Dorfin immunoprecipitation assay, binding to the ICD. Binding to deglycosylated CaSR suggests that Dorfin-mediated proteasomal degradation occurs in the ER.[223] Although Dorfin was not identified in the CaSR interactome, another important E3 ubiquitin ligase involved in the Hsp90-dependent ubiquitination mechanism, chromatin immunoprecipitation, was identified as a CaSR interactor.[184] The degradation of CaSR can also occur in lysosomes, where a deubiquitinating enzyme specific for K63- ubiquitin linkages and an associated molecule with the SH3 domain of STAM (AMSH) were

both reported to target CaSR for lysosomal degradation. The C-terminal tail of CaSR is rich in PEST, which also promotes lysosomal pathways. Endocytosis may also promote CaSR recycling to the plasma membrane, via a Rab11a-dependent mechanism.[175,224,225]

Other adapter proteins present in the interactome include filamin A and filamin B. Filamin A has been reported to interact with CaSR C-terminal and target the receptor to sites of Rho GTPase activity. Notably, CaSR induces ERK activity only in the presence of filamins.[220–223] Rho and PI 4-kinase β were also enriched in the Co-IP complex with CaSR, suggesting an importance of this interaction in inositol lipid synthesis.[173] β1-containing integrin oligomers also interact with CaSR and contribute to cellular adhesion and migration.[226] CaSR interacts with potassium channel proteins Kir4.1 and Kir4.2 in an inhibitory manner. This was determined experimentally using a yeast two-hybrid system and co-immunoprecipitation.[174] However, the two most notable protein families identified by CaSR-PPI include VAMP/synaptobrevin associated proteins, or VAMP-associated proteins (VAPs), and the Rab small-GTPase family. These families were enriched in their protein expression in the presence of high Ca^{2+} concentrations and have been associated with CaSR activation. These proteins will be discussed in the context of their known functions and the relationship of those functions to CaSR-dependent cell signaling (Table 2).

4.4 | VAPs: highly Ca^{2+}-dependent CaSR interactors

VAPs are highly conserved ER membrane proteins found in various eukaryotic cells. VAPA and VAPB share significant sequence similarity and a similar primary structure.[227,228] These proteins form dimers with themselves,[229] each other, and both VAMP1/VAMP2,[230] which is accomplished primarily via the VAPA/VAPB TMD and coiled-coil domains. Their intrinsically disordered regions in both proteins facilitate structural flexibility,[231] especially in the cytosolic regions. They have been implicated in ER contacts with multiple organelles, including the mitochondria,[232] plasma membrane,[233] peroxisomes,[234] Golgi apparatus,[235] and both early [236] and late endosome/lysosome membranes.[237] Over 250 proteins have been reported to interact with VAPA and/or VAPB, and investigation of their interactome reveals that the two share ~50% of all the interacting proteins.[238] Notably, the predominant portion of these shared proteins all exhibit small linear motifs known as FFAT (two phenylalanines in an acidic tract).[227,238,239] While this is the primary domain facilitating protein-protein interactions, not all proteins interact with VAP proteins via FFAT motif recognition, as there is evidence that the coiled-coil domain,[240] the single-pass TMD,[240–242] and the C-terminus[243] may be involved in binding of VAPA/VAPB to its partners. There is also evidence that the primary binding domains of VAPs are cleaved,[244] which adds more complexity to its interactions with proteins. To date, the specific interaction site of VAPs with CaSR remains unclear.

VAPs reduce the energetic barrier for transferring lipids between membranes with the help of lipid-binding proteins, which often contain FFAT motifs. VAPs are essential for Golgi-mediated transport. Suppression of VAPs through RNA interference decreases phosphatidylinositol-4-phosphate (PI4P), DAG, and sphingomyelin abundance, within the Golgi membranes. This outcome subsequently hinders transport processes orchestrated by the Golgi apparatus.[245] Remarkably, the overexpression of the wild-type A isoform (VAPA), but not the B isoform (VAPB), exerts an inhibitory effect on the ER-to-Golgi transport of membrane proteins. This inhibition was attributed to VAPA's ability to impede the integration of the anterograde membrane cargo vesicular stomatitis virus glycoprotein into ER vesicles.[235]

Lipid-binding proteins, such as the oxysterol-binding proteins (OSBPs) and the OSBP-related proteins (ORPs), are recruited to the ER by interacting with VAPA and VAPB through their FFAT motifs. OSBPs also exhibit an additional capability to target the trans-Golgi network, primarily through a distinct domain known as the PI4P-binding pleckstrin homology (PH) domain.[246–249] Other ER-Golgi membrane contact sites are also coordinately mediated by the ceramide transfer protein (CERT), and ER-plasma membrane contacts are mediated by FFAT-containing Nir proteins. Both CERT and Nirs feature FFAT motifs that facilitate their interaction with the VAPs, while CERT is equipped with FFAT and PH domains.[245,250,251]

Both VAPA and VAPB were detected in the presence of 4 mmol/L extracellular calcium (Ca^{2+}) in the interactome by Gorkhali et al.,[184] where VAPA was identified as the most Ca^{2+}-dependent interactor with a 43-fold enrichment, whereas VAPB exhibited 4- to 8-fold enrichment. The interrelationship between VAPA and CaSR was further confirmed by high-resolution confocal microscopy-based pixel intensity correlation analysis in the same study. The discovery of VAPs as CaSR interactors holds substantial significance, given the critical role of lipids and sterols (most notably cholesterols to GPCRs) as positive-allosteric modulators.[252,253] Structures of human CaSR determined by Cryo-EM revealed the presence of several cholesterols within the TMD of CaSR, notably situated between TM6 and TM7. Removal of these cholesterols by methyl-β-cyclodextrin leads to a decreased receptor basal activity, as well as reduced efficacy and potency of Ca^{2+}.[112] Although direct evidence of VAP involvement in the ER-to-golgi transport of CaSR is lacking, research by Gorkhali et al.[184] demonstrated an interdependent relationship between CaSR and

TABLE 2 CaSR interacting proteins.

Interacting protein	Method	CaSR domain	Function	Paper DOI
AP2	Co-IP	N/A	Endocytosis	https://doi.org/10.1093/hmg/ddab076
AMSH	Y2H, GST	Residue 895-1075	Deubiquitinating	https://doi.org/10.1016/j.bbrc.2006.06.169
β-arrestins	Y2H, Co-IP	Carboxyl terminus	Trafficking/signaling	https://doi.org/10.1210/me.2004-0450
Cavs	Co-IP	Intracellular loops 1 and 3	Structural/scaffolding	https://doi.org/10.1042/BJ20111277
CNX	Co-IP, Colocalization	N/A	Chaperone	https://doi.org/10.1038/s41598-021-00067-2
CaM	Co-IP, GST	Residue 871-898	Trafficking/signaling	https://doi.org/10.1074/jbc.M110.147918
Dorfin	Co-IP	Residue 880-900	Ubiquitinating	https://doi.org/10.1074/jbc.M513552200
Filamin A	Y2H, Co-IP	Residue 906-980	Rho/ERK	https://doi.org/10.1210/en.2002-220240 https://doi.org/10.1074/jbc.M100775200
GABA	Co-IP	ECD	cGPCR	https://doi.org/10.1210/en.2007-0653 https://doi.org/10.1074/jbc.M700924200
GRP78	Co-IP	N/A	Chaperones	https://doi.org/10.1038/s41598-021-00067-2
Integrins	Co-IP	N/A	Cell migration	https://doi.org/10.1074/jbc.M111.265454
Kir4	Y2H, Co-IP	Carboxyl terminus	K channels	https://doi.org/10.1152/ajprenal.00269.2006
mGluR	Co-IP		cGPCR	https://doi.org/10.1074/jbc.M105662200
p24A	Y2H, Co-IP	Carboxyl terminus	Trafficking	https://doi.org/10.1016/j.bbrc.2010.03.156
PI 4-kinase	Co-IP	N/A	Signaling	https://doi.org/10.1074/jbc.M200831200
RAMP1	Co-IP	N/A	Trafficking	https://doi.org/10.1242/jcs.02598
RAMP3	Co-IP	N/A	Trafficking	https://doi.org/10.1242/jcs.02598
Rabs	Co-IP, Function	N/A	Trafficking	https://doi.org/10.1210/en.2010-0422 https://doi.org/10.1210/me.2006-0523 https://doi.org/10.1038/s41598-021-00067-2
Rho	Co-IP	N/A	Signaling	https://doi.org/10.1074/jbc.M200831200
VAPA	Co-IP	N/A	Trafficking	https://doi.org/10.1038/s41598-021-00067-2 https://doi.org/10.1126/scisignal.2002208
14-3-3	Co-IP	R890RxxxxRKR898	ER retention	https://doi.org/10.1371/journal.pone.0136702 https://doi.org/10.1159/000320560

Abbreviations: AMSH, associated molecule with the SH3 domain of STAM; AP2, adaptor protein-2; CaM, calmodulin; Cavs, caveolins; cGPCR, family C G-protein-coupled receptor; CNX, calnexin; Co-IP, co-immunoprecipitation; ECD, extracellular domain; ER, endoplasmic reticulum; GABA, γ-aminobutyric acid; GRP78, glucose-regulated protein 78; mGluR, metabotropic glutamate receptor; N/A, not available.

VAPA during Ca^{2+}-induced enhancement of near-cell membrane expression. This suggests that VAPA likely contributes to the forward trafficking of CaSR in response to Ca^{2+} stimulation.

VAPs also bind to several other cytoplasmic proteins and contribute to Ca^{2+} exchange, in addition to lipid and ceramide transfer proteins. The interaction of VAPB on the ER with mitochondrial outer membrane protein tyrosine phosphatase-interacting protein-51 was found to promote ER-mitochondria contact sites and Ca^{2+} exchange. Impaired uptake of ER-released Ca^{2+} by the mitochondria was observed with depletion of either protein.[254] VAP-mediated contact sites also play roles in governing the exchange of Ca^{2+} between the ER and lysosomes. Notably, the interaction between VAPA and the steroidogenic acute regulatory protein-related lipid transfer domain-3, STARD3, is instrumental in the process of forming ER-endolysosome contact sites. Additionally, cholesterol sensor ORP1L contacts VAPs to control late endosome positioning and cholesterol transfer between the ER and endolysosome.[237,255–258] VAPA and VAPB also interact with autophagy-associated proteins FIP200 and ULK1 via FFAT-mediated interactions, thereby modulating the process of autophagosome biogenesis.[259] In summary, it is conceivable that the interaction between VAPs and CaSR may regulate CaSR-mediated Ca^{2+} dynamics and the endocytosis of CaSR.[260–264]

4.5 | Rabs: G-protein switches involved in CaSR expression and activity

As previously described, CaSR also fulfills a pivotal role in the secretion of hormones, various cytokines and chemokines, growth factors, and neurotransmitters.[260–264] A notable finding of Gorkhali et al.'s[184] CaSR interactome study was the interaction of multiple Rab small GTPase proteins with CaSR. Rabs are involved in consecutive transport stages including vesicle formation, vesicle and organelle motility, and tethering of vesicles with target membranes.[265] Specifically, Rab5, Rab6, and Rab18 were all three enriched in the interactome.[184] Expression of different Rab proteins may be associated with different timeframes in the forward trafficking and endosomal pathway. For example, the transition from Rab5 to Rab7 has been described as a transitional event, wherein an endosome moves from the early endosomal pathway to the late endosomal pathway.[266] Interestingly, the functions of CaSR interactors, specifically VAP proteins, have been associated with certain Rab proteins, like Rab5 and Rab7. When VAPs were genetically knocked out, an accumulation of Rab5 and Rab7 was observed, which coincided with increased phosphatidylinositol-4-phosphate levels in the Golgi.[267]

The Rab1 protein was reported to be responsible for trafficking of CaSR, particularly from ER to Golgi.[268] Rab1 coordinates with Sar1 in this trafficking mechanism.[268] CaSR expression was strongly augmented by co-expression with Rab1 and was attenuated by disruption of endogenous Rab1 or Sar1 function.[268,269] Known CaSR interactors Rab1, Rab11a, and Rab27b were not present in the CaSR interactome, perhaps due to transient interactions with CaSR. It has been reported that the agonist-induced endocytosis and recycling of CaSR affects the secretion of PTH-related peptide (PTHrP). CaSR is constitutively endocytosed and recycled to the plasma membrane by a Rab11a-dependent mechanism. This trafficking of CaSR facilitates the secretion of PTHrP via endosomal pathways. However, the presence of AMSH, a CaSR-binding protein, alters CaSR recycling and leads to reduced CaSR expression and decreased PTHrP secretion.[224] In addition, vesicular trafficking of CaSR reportedly regulates the secretion of various cytokines and chemokines including IL-6, IL-1β, IL-8, IL-10, and chemokine (CC-motif) ligand 2/monocyte chemoattractant protein-I, and this process is tightly related to Rab27b. CaSR promotes Rab27b expression and drives Rab11a-dependent coupling of recycling endosomes to secretory vesicles.[261,270]

The association of VAP proteins with Rab proteins has been observed to play a role in some virologic mechanisms. For example, a complex of VAPA, ORP3, and Rab7 was found to mediate human immunodeficiency virus 1-induced nuclear invaginations.[271] Knockdown of VAPB, Rab11b, and Rab18, simultaneously reduced the titers of released herpes simplex virus 1 (HSV-1) virus with statistical significance, while also leading to the nuclear accumulation of encapsidated particles.[272] Thus, evidence suggests that VAPB, together with Rab11b and Rab18, contributes to the virulence of HSV-1. These findings in the CaSR interactome further suggest that these two families of CaSR interactors may synergize to regulate cellular defense mechanisms against pathogens, such as viruses.

5 | TRANSLATIONAL POTENTIAL OF TARGETING CASR FOR HUMAN DISEASES

The recent advent of cryo-EM has ushered in a new era in structural and functional studies of GPCRs, especially for CaSR. This new structural evidence in biochemical and structural determination of the framework of CaSR and its interaction sites with natural ligands, as well as exogenous PAMs and NAMs, further support a coactivation model involving calcium and amino acids for functional cooperativity and provide molecular insights into disease-related mutations. These key structural determinants in ligand interaction and regulation have the potential to promote rapid development of novel drugs against various calciotropic and noncalciotropic diseases.[55,111,273,274]

Newly developed Ca^{2+} sensors, such as R-CatchER and G-CatchER$^+$, are capable of capturing rapid Ca^{2+} dynamics in the ER. These new tools will improve our understanding of CaSR-mediated Ca^{2+} signaling, the molecular basis of diseases, and functional cooperativity of ligands, as well as facilitate drug discovery.[275,276] Using R-CatchER, we have reported the first direct observation of ER Ca^{2+} oscillations, which directly link the extracellular, cytosolic, and ER compartments mediated by CaSR.[275,276] We are further able to detect that extracellular Ca^{2+} and agonists, such as L-Phe and TNCA, cooperatively tune ER Ca^{2+} oscillations mediated by CaSR. Importantly, for the first time, we have shown that disease mutations largely alter ER Ca^{2+} responses, oscillation frequency, and cooperativity.[109,111] These exciting results largely support our co-activation working model based on our structural determination (Figure 3B).[106,108,111] R-CatchER will also be invaluable in elucidating the molecular mechanisms mediated by CaSR and other GPCRs that integrate Ca^{2+} signaling. By expanding our capability to visualize Ca^{2+} dynamics, R-CatchER can be further applied to drug discovery for human diseases related to ER dysfunction and Ca^{2+} mishandling.

The establishment of the first CaSR PPI network has significantly extended the repertoire of the CaSR interactome and has revealed likely novel players and pathways of CaSR participation in Ca^{2+} ER dynamics. This will lead to improved understanding of CaSR in

respect to agonist-mediated ER-protein processing, surface expression, endocytosis, degradation, and signaling pathways.[109,275] When inserted into the plasma membrane, CaSR primarily interacts with other proteins through interactions in the ICD loops and the C-terminal tail. Characterizing protein-protein interactions during CaSR folding and trafficking, however, is less understood. Future work on the role of identified novel CaSR-mediated proteins is expected to provide new insights into the molecular basis of diseases caused by CaSR mutations and dysregulated CaSR activity caused by its protein interactors and functional cooperativity.

One area where the structural nature of CaSR remains a mystery is in the cytosolic C-terminal domain, which includes multiple interaction sites for downstream signaling. A recent CryoEM structure of CaSR in complex with the G_q heterotrimer demonstrated binding dynamics that resemble, but are still distinct from, the interaction between G_i and mGluR2[277,278] and mGluR4.[278] ICL3, along with residues adjacent to the cytosol in TM3 and TM4, accommodates the a5 helix of the α-subunit of G_q upon binding.[125] The C-terminal structural complex of CaSR with other G-protein heterotrimers, or β-arrestin, may also be determined using NanoBiT technology, based on its recent success in determining CaSR structures with G_q (PDB ID: 8SZG, 8WPU), G_i (PDB ID: 8SZH, 8SZI), and several other GPCR structures, including VIP1R, a class B GPCR, with the G_s heterotrimer, and PACAP27[279]; the histamine H_1 receptor with the G_q heterotrimer[280]; and the thyrotropin-releasing hormone receptor with G_q.[281,282] Manipulating experimental CryoEM conditions with lipid nanodiscs (PDB ID: 8SZF-I) and structure-stabilizing nanobodies (PDB ID: 7E6U[113]) has also become increasingly more common in recent determinations of CaSR membrane structures.[113,124] However, as use of these techniques becomes more common, attention must be paid to possible steric-dependent artefacts that deviate from the native structure of the protein, which may obscure key structural determinants of CaSR activity.[124,283,284]

Lastly, some of the mystery in resolving the C-terminal domain of CaSR, or the binding modes of various families of G-protein heterotrimers, may be improved by implementing techniques that correct or compensate for the membrane disruptions that are inherent to the use of detergent micelles in sample preparation for CryoEM. The broader understanding of the C-terminal domain's role in G_q recognition by CaSR, recently gained through NanoBiT functionalization, recapitulates this idea.[124] Further improvement in resolution, data fitting, modeling calculation, and native conditions may also resolve the debate between symmetric and asymmetric activation of CaSR.[285–287] The translational value of elucidating the mechanisms involved in regulating CaSR cell surface expression and biased signaling cannot be overstated. Further discoveries and advances in rapid subcellular Ca^{2+} sensors, structure-stabilizing techniques, and computational medicine will facilitate improved patient outcomes at the bedside for various calciotropic and noncalciotropic diseases related to CaSR pathology.

AUTHOR CONTRIBUTIONS

Li Tian, Corey Andrews, Qiuyun Yan, and Jenny J. Yang wrote the manuscript and participated in literature collection.

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CONFLICT OF INTEREST STATEMENT

Jenny J. Yang holds shares in the company InLighta Biosciences LLC. Jenny J. Yang is a named inventor on issued or pending patents related to Calcium-sensing Receptor (US 10639299 B2). The authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data used to support this review are included within the article.

ETHICS STATEMENT

None.

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