Homer1 mediates CaSR-dependent activation of mTOR complex 2 and initiates a novel pathway for AKT-dependent β-catenin stabilization in osteoblasts

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Running title: Homer1 links CaSR to mTORC2

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Keywords: calcium-sensing receptor (CaSR), Homer1, mechanistic target of rapamycin complex 2 (mTORC2), AKT Ser/Thr kinase, osteoblast, cell signaling, bone formation, β-catenin, family C G protein coupled receptor

ABSTRACT

The calcium-sensing receptor (CaSR) is critical for skeletal development, but its mechanism of action in osteoblasts is not well characterized. In the central nervous system (CNS), Homer scaffolding proteins form signaling complexes with two CaSR-related members of the G protein–coupled receptor (GPCR) family C, metabotropic glutamate receptor 1 (mGluR-1) and mGluR-5. Here, we show that CaSR and Homer1 are co-expressed in mineralized mouse bone and also co-localize in primary human osteoblasts. Co-immunoprecipitation experiments confirmed that Homer1 associates with CaSR in primary human osteoblasts. The CaSR–Homer1 protein complex, whose formation was increased in response to extracellular Ca2+, was bound to mTOR complex 2 (mTORC2), a protein kinase that phosphorylates and activates AKT Ser/Thr kinase (AKT) at Ser473. siRNA-based gene-silencing assays with primary osteoblasts revealed that both CaSR and Homer1 are required for extracellular Ca2+-stimulated AKT phosphorylation and thereby inhibit apoptosis and promote AKT-dependent β-catenin stabilization and cellular differentiation. To confirm the role of the CaSR–Homer1 complex in AKT initiation, we show that in HEK-293 cells, co-transfection with both Homer1c and CaSR, but neither with Homer1c nor CaSR alone, establishes sensitivity of AKT-Ser473 phosphorylation to increases in extracellular Ca2+ concentrations. These findings indicate that Homer1 mediates CaSR-dependent AKT activation via mTORC2 and thereby stabilizes β-catenin in osteoblasts.

The CaSR is a Family C G-protein coupled receptor (GPCR) critical for the development of bone and cartilage (1-3) and contributes to the development of the CNS, in part by promoting the differentiation of oligodendrocytes and formation of cerebral white matter (4). When CaSR exon-7, encoding the receptor’s heptahedral domain and intracellular C-terminus, was selectively knocked out of early osteoblast lineage cells in mice, bone mass greatly reduced (1,2) and selective deletion in chondrocytes was embryonic lethal (1). In a human case of neonatal severe hyperparathyroidism, in which early stop codons were present in both CaSR alleles, additional phenotypic disturbances included growth retardation, generalised osteopenia, and attenuated development of the cerebral white matter (5). The signaling pathways that underlie CaSR-mediated control of cell fate, however, are largely undefined.

We have previously reported that activation of CaSR results in the dual phosphorylation of AKT in osteoblasts (6).
Phosphorylation of AKT at Thr\textsuperscript{308} is mediated by 3-phosphoinositide-dependent kinase 1 (PKD1) (7,8) downstream of phosphatidylinositol 3-kinase (PI3K), whereas phosphorylation of AKT at Ser\textsuperscript{473} is mediated by the mechanistic target of Rapamycin (mTOR) complex-2 (mTORC2), also known as PDK2 (9-11), a complex of proteins that specifically includes rapamycin-insensitive companion of mTOR (Rictor), but not the regulatory associated protein of mTOR (Raptor) (12). mTORC2-dependent Ser\textsuperscript{473} phosphorylation of AKT occurs independently of, and is required for, Thr\textsuperscript{308} phosphorylation (9). The upstream mechanism by which mTORC2 couples to the CaSR, however, is unknown. Recently it was shown that mTORC2 localizes to the plasma membrane, independently of PI3K, where it functions to phosphorylate AKT at Ser\textsuperscript{473} (13).

Canonical Wnt signaling in osteoblasts is a key determinant of bone cell fate and function (14,15). The process is dependent on the binding of extracellular Wnt proteins, such as Wnt3a, to Frizzled-LDL receptor-related protein-5&6 (LRP5/6), inactivating glycogen synthase kinase-3 (GSK3) via plasma membrane sequestration, resulting in the stabilization of cytoplasmic β-catenin which subsequently translocates to the nucleus where it promotes expression of genes under the control of TCF/LEF transcription factors (16). Independently of canonical Wnt-dependent inhibition of GSK3, AKT inhibits the kinase activity of GSK3 via direct phosphorylation at Ser\textsuperscript{21} (α-subunit) (17) and Ser\textsuperscript{9} (β-subunit) (18). Similarly, direct phosphorylation of β-catenin at Ser\textsuperscript{552} by AKT promotes its nuclear translocation and transcriptional activity (19).

We previously reported that activation of the CaSR in human osteoblasts resulted in the AKT-dependent translocation of β-catenin to the nucleus in primary human osteoblasts, thus potentially linking the AKT and Wnt/β-catenin pathways in bone cells (6). In agreement, Wnt3a-dependent activation of LRP5/6 in stromal ST2 cells activated the mTORC2/AKT pathway and promoted cell differentiation (20) – showing that cross-talk between the pathways can potentially occur in both directions. However, the underlying mechanism by which the CaSR links to the mTORC2/AKT pathway in bone cells has not been defined.

Homer1 links CaSR to mTORC2

Homer proteins act as scaffolds at the plasma membrane for the assembly of signaling complexes that include the family C metabotropic glutamate receptors mGluR1 and mGluR5 (21-23). The outcomes include cell fate determination via AKT (22,24) and ERK\textsubscript{2} (23), and underlie neuroplasticity in the hippocampus and other areas of the CNS (25). Several Homer isoforms are recognized (26,27). Among these, the long isoforms Homer1b/c (but not the short isoform 1a), as well as Homer2 and -3 contain coiled-coil domains that mediate self-multimerization, and Ena/Vasp homology (EVH1) domains (present in all Homer isoforms) that mediate signaling interactions (28,29). Disruption of Homer-mGluR1 interactions in mouse neurons decreased PI3K activity (24). Whether, in some cell contexts, Homer also supports CaSR-dependent activation of mTORC2 upstream of AKT is unknown.

In the present study we tested the hypothesis that in osteoblasts the long isoforms of Homer1 (hence referred to as Homer1) containing coiled-coil regions may link CaSR to mTORC2, resulting in activation of AKT and β-catenin stabilization in these cells (hypothesis shown schematically in Figure 1).

Results

CaSR and Homer1 mediated extracellular Ca\textsuperscript{2+}-stimulated AKT signaling in osteoblasts.

We first confirmed that activation of CaSR by its physiological ligand Ca\textsuperscript{2+} resulted in dual phosphorylation of AKT with phosphorylation of Ser\textsuperscript{473} preceding phosphorylation of Thr\textsuperscript{308} (Figure 2A). Maximal phosphorylation at these sites was reached 15 min following exposure to elevated Ca\textsuperscript{2+} and was the time point used for all subsequent signaling analyses (Figure 2A). Inhibition of mTORC2 with Torin1 decreased Ca\textsuperscript{2+}-stimulated phosphorylation of AKT at both Ser\textsuperscript{473} and Thr\textsuperscript{308} in a concentration dependent manner (Figure 2B). We confirmed that Torin1 treatment had no effect on mTORC1 at this early time point as evidenced by no change in mTORC1-Ser\textsuperscript{2448} phosphorylation, a marker of mTORC1 activity (30) (Figure 2B). Therefore changes in AKT phosphorylation in response to the inhibitor at this time point were not due to a previously reported phenomenon of negative-feedback dependent regulation of mTORC2 by mTORC1 (31,32), but due to
inhibition of mTORC2 protein kinase activity per se.

We next hypothesized that Homer1 may mediate extracellular Ca\textsuperscript{2+}-induced mTORC2-dependent AKT signaling downstream of the CaSR in osteoblasts. Consistent with this hypothesis, when the expression of either the CaSR (Figure 2C-D) or Homer1 (Figure 2E-F) proteins was reduced selectively by specific siRNA in human osteoblasts, we observed decreased Ca\textsuperscript{2+}-dependent AKT phosphorylation at both Ser\textsuperscript{473} and Thr\textsuperscript{308} compared to control.

**Homer1 and CaSR co-localized and co-immunoprecipitated in vitro with mTOR and the mTORC2 specific protein Rictor in response to elevated extracellular Ca\textsuperscript{2+}.**

Deconvolution microscopy of mouse bone sections revealed that CaSR and Homer1 were expressed in the same cells of mineralized bone (Figure 3A top row). As a comparison CaSR(-/-) mineralized bone sections are also shown (Figure 3A, bottom row). Confocal microscopy of a monolayer culture of primary human osteoblasts stained for CaSR and Homer1 is shown in Figure 3B (top row). A high-resolution image of a single osteoblast (Figure 3B, bottom row) indicated co-localization (Pearson’s coefficient = 0.41; below threshold = -0.02).

Since Homer1 was required for extracellular Ca\textsuperscript{2+}-dependent dual phosphorylation of AKT (Figure 2E-F), we hypothesized that Homer1 may interact with the CaSR and mTORC2 protein components, and also tested whether any interaction might be dependent on an increase in extracellular Ca\textsuperscript{2+} concentration. After immunoprecipitation with an anti-Homer1 antibody, Homer1 was itself readily detected and this was unaffected by an increase in extracellular Ca\textsuperscript{2+} concentration from 1.0 to 3.5 mM (Figure 3C, 3D ns diff). Homer1 protein from non-immunoprecipitated lysate is shown at the same molecular weight (~45 kDa, Figure 3C). CaSR protein, at ~170 kDa – a molecular weight representing glycosylated, plasma membrane associated CaSR (33) (Uniprot ID P41180), was detected in Homer1 immunoprecipitates and the amount of CaSR bound to Homer1 increased in response to elevated Ca\textsuperscript{2+} (Figure 3C, 3D p<0.001). CaSR protein was present at 170 kDa in the osteoblast lysate (Figure 3C, “CaSR”) and also at a lower molecular weight of ~120 kDa (Figure 3C, “#”). This 120 kDa isoform corresponds to non-glycosylated ER/Golgi associated CaSR and was not immunoprecipitated with Homer1 (Figure 3C).

mTOR and the mTORC2-specific protein Rictor were present in non-immunoprecipitated osteoblast lysates (Figure 3C) and were detected in Homer1 immunoprecipitates. In the case of mTOR, however, no Ca\textsuperscript{2+}-dependent increase in its level was observed (Figure 3C, 3D ns diff), whereas Rictor protein levels, like those of the CaSR, increased in response to elevated extracellular Ca\textsuperscript{2+} (Figure 3C, 3D p<0.05). On the other hand, although the mTORC1 specific protein Raptor was present in lysates (Figure 3C), it was not detected in Homer1 immunoprecipitates at either control or elevated Ca\textsuperscript{2+} (Figure 3C). Thus, in the presence of an elevated extracellular Ca\textsuperscript{2+} concentration, we observed increased association between the CaSR, Homer1 and the mTORC2-specific protein Rictor, but not the mTORC1-specific protein Raptor.

**CaSR and Homer1 were required for extracellular Ca\textsuperscript{2+}-dependent β-catenin nuclear translocation following AKT activation in osteoblasts.**

We previously reported that the CaSR agonist strontium (Sr\textsuperscript{2+}) induced nuclear translocation of β-catenin in human osteoblasts following activation of AKT and demonstrated that these effects were mediated by the CaSR (6). Consistent with this observation, exposure of cells to elevated extracellular Ca\textsuperscript{2+} resulted in concentration-dependent phosphorylations of the inhibitory sites of GSK3α/β at Ser\textsuperscript{9}/Ser\textsuperscript{9} respectively and activatory site of β-catenin at Ser\textsuperscript{552} (Figure 4A), and nuclear translocation of β-catenin, as demonstrated by western blotting of nuclear fractions at various times over 60 min (Figure 5Ai). The phosphorylation of mTOR at Ser\textsuperscript{2448} (carried out by p70S6K and indicative of mTORC1 activation downstream of AKT (30)) was also stimulated by elevated extracellular Ca\textsuperscript{2+} (Figure 4A).

Osteoblasts transfected with siRNA directed against the CaSR exhibited decreased CaSR protein expression (Figure 4Bi, bottom row, 4Bi, p<0.05) and knockdown of the CaSR attenuated the stimulatory effect of elevating extracellular Ca\textsuperscript{2+} on the phosphorylations of AKT-Ser\textsuperscript{473} (p<0.01), AKT-Thr\textsuperscript{308} (p<0.05) GSK3α/β-
Ser\textsuperscript{21,Ser}\textsuperscript{9} (p<0.01), β-catenin-Ser\textsuperscript{552} (p<0.05), and mTOR-Ser\textsuperscript{2448} (p<0.01) (Figure 4Bi for western & 4Bii for significance) as well as nuclear localization of β-catenin (Figure 5Aii, 5Aiv p<0.01).

Osteoblasts transfected with siRNA directed against Homer1 exhibited decreased Homer1 protein expression (Figure 4Ci, bottom row, 4Cii p<0.01) and knockdown of Homer1 also attenuated the stimulatory effect of elevated extracellular Ca\textsuperscript{2+} on the phosphorylations of AKT-Ser\textsuperscript{473} (p<0.05), AKT-Thr\textsuperscript{308} (p<0.01), GSK3α/β–Ser\textsuperscript{21}/Ser\textsuperscript{9} (p<0.01), β-catenin-Ser\textsuperscript{552} (p<0.01), and mTOR-Ser\textsuperscript{2448} (p<0.05) (Figure 4Ci for western & 4Cii for significance) as well as nuclear translocation of β-catenin (Figure 5Aiii, 5Av p<0.01).

Taken together, these observations indicate that the CaSR and Homer1 were required for extracellular Ca\textsuperscript{2+} stimulation of an AKT-dependent pathway that controlled β-catenin translocation. To separately confirm the role of AKT on the nuclear translocation of β-catenin in vitro, chemical inhibition of AKT with AKT-XI (20 μM) reduced osteoprotegerin (OPG) expression – a marker of the LEF/TCF pathway (15) (Figure 5vi). A degree of CaSR control of β-catenin stabilization and translocation was also apparent in vivo as CaSR (-/-) mice had reduced protein levels of the Wnt marker OPG compared to wt (Figure 5B). Similarly, qPCR analysis also showed that the mRNA levels of several Wnt responsive genes in CaSR (-/-) mice were reduced compared to wt (Table 1).

As a control for the signaling analysis and to assess potential off-target effects, siRNA was used to knockdown Ga\textsubscript{12}, a protein that does not affect AKT but inhibits ERK\textsubscript{1/2} in osteoblasts (34,35). Silencing Ga\textsubscript{12} resulted in reduced Ga\textsubscript{12} protein (Figure 4Di bottom row, 4Dii p<0.001) and, as expected, further increased Ca\textsuperscript{2+}-induced ERK\textsubscript{1/2} phosphorylation (Figure Supp 1). However, knockdown of Ga\textsubscript{12} had no effect on the phosphorylations of AKT at Ser\textsuperscript{473} or Thr\textsuperscript{308} or of its key downstream targets including GSK3, β-catenin or mTORC1 as revealed by p70 S6K-dependent phosphorylation of mTOR at Ser\textsuperscript{2448} (Figure 4Di for western, 4Dii for significance).

**Homer1 links CaSR to mTORC2**

**CaSR and Homer1 were required for extracellular Ca\textsuperscript{2+}-dependent AKT and GSK3β phosphorylation in MG63 osteosarcoma cells**

The effect of Homer1 and CaSR on the phosphorylation of AKT-Ser\textsuperscript{473} and GSK3β-S\textsuperscript{9} in response to elevated extracellular Ca\textsuperscript{2+} was also examined in a second osteoblast cell type – the commonly used osteosarcoma cell line MG63. We found that Homer1 and CaSR were expressed in MG-63 cells (Figure 6). In these cells both AKT-Ser\textsuperscript{473} and GSK3β-S\textsuperscript{9} phosphorylation were increased in response to elevated extracellular Ca\textsuperscript{2+}, and siRNA-dependent knockdown of either Homer1 or CaSR clearly reduced the phosphorylation level of both proteins in response to Ca\textsuperscript{2+}o (Figure 6). These findings were consistent with our analyses performed in primary human osteoblasts (Figure 4).

**Effect of CaSR and Homer1 on cell fate of osteoblasts**

Given the observed effect of the CaSR and Homer1 on Ca\textsuperscript{2+}-stimulated nuclear β-catenin translocation (Figure 5), which promotes differentiation in osteoblasts (15) we next investigated whether the CaSR and Homer1 modulated alkaline phosphatase (ALP) activity, another marker of osteoblast differentiation (36). Elevated extracellular Ca\textsuperscript{2+} (1.0 - 2.0 mM) stimulated ALP activity by 4 to 5-fold after 3 d (Figure 7A, p<0.01). Silencing the CaSR abolished the stimulatory effect of elevated Ca\textsuperscript{2+} (2.0 mM) on ALP activity but had no effect on basal ALP activity (Figure 7B). Silencing Homer1 suppressed basal ALP activity in cells exposed to control extracellular Ca\textsuperscript{2+} (1.0 mM, p<0.05) and abolished the response to elevated Ca\textsuperscript{2+} (2.0 mM) (Figure 7C), demonstrating that Homer1 supports a key function of differentiated osteoblasts in the physiologically relevant extracellular Ca\textsuperscript{2+} concentration range.

Finally we investigated the roles of CaSR and Homer1 in AKT-dependent osteoblast survival in the presence of pro-apoptotic oxidative stress, an important determinant of viability in vivo (37), using caspase-3 activity as the readout. Elevated extracellular Ca\textsuperscript{2+} suppressed oxidative stress-induced elevations of caspase-3 activity in a concentration-dependent manner from 1-2 mM (Figure 7D) and the pro-survival effect of elevated Ca\textsuperscript{2+} was reversed upon silencing either the CaSR...
(Figure 7E) or Homer1 (Figure 7F). Furthermore, downstream of CaSR and Homer1, mTORC2-dependent phosphorylation of AKT-Ser$^{473}$ (9) was clearly critical in this action of extracellular Ca$^{2+}$, since the inhibitor Torin1 caused significant concentration-dependent increases in caspase-3 activity in the presence of 2 mM Ca$^{2+}$ and the oxidative stressor 125 μM H$_2$O$_2$ (Figure 7G), while wortmannin, the inhibitor of PI3K upstream of AKT, had no effect (Figure 7H).

Transfection of Homer-1c into HEK-293 cells stably expressing CaSR established AKT-Ser$^{473}$ sensitivity to extracellular Ca$^{2+}$

It is possible that the CaSR-Homer1-mTORC2 complex identified in osteoblasts in the present study operates more widely in other cell types that express both the CaSR and Homer1. To test this hypothesis we performed experiments in the human embryonic kidney cell line, HEK-293, and HEK-293 cells stably expressing CaSR (HEK-CaSR), a well-established cellular system for studies of CaSR signaling (38,39).

Transient transfection of control HEK-293 cells with Homer1-c (HEK-Homer1c) resulted in maximal Homer1c protein expression 48 h post transfection (Figure 8A). Exposure of control HEK-CaSR cells to various Ca$^{2+}$o concentrations in the range of 0.1-5 mM for 15 min yielded no increase in AKT-Ser$^{473}$ phosphorylation (Figure 8B). Similarly, HEK-293 cells transiently transfected with only Homer1c (in the absence of CaSR) failed to respond to raised extracellular Ca$^{2+}$ with AKT-Ser$^{473}$ phosphorylation (Figure 8C). However, when HEK-CaSR cells were transiently transfected with Homer1c, Ca$^{2+}$o-dependent AKT-Ser$^{473}$ sensitivity was observed (Figure 8D). Therefore, both Homer1c and CaSR were required for Ca$^{2+}$o-stimulated AKT-Ser$^{473}$ phosphorylation in HEK-293 cells.

Summary of Results

We made several novel observations in this study. Homer1 protein was expressed in human osteoblasts. In mineralized mouse bone sections of wt mice, high levels of CaSR expression were associated with high levels of Homer1 expression. In agreement with this observation, confocal microscopy in primary human osteoblasts demonstrated co-localization of Homer1 and CaSR. Homer1 and the mature, glycosylated, isoform of CaSR were shown to exist in a protein complex with mTOR and the mTORC2-specific protein Rictor by immunoprecipitation, and the level of CaSR and Rictor protein in the complex with Homer1 was significantly increased in response to increased extracellular calcium. Homer1 and CaSR modulated Ca$^{2+}$-induced AKT phosphorylation, and attendant translocation of β-catenin to the nucleus within 15 minutes of treatment. In MG63 osteosarcoma cells, which expressed both CaSR and Homer1, phosphorylation of AKT-Ser$^{473}$ and GSK3β-S$^{9}$ was increased in response to extracellular Ca$^{2+}$, while siRNA directed at either Homer1 or CaSR attenuated these effects. As predicted from the above signaling studies, both Homer1 and CaSR positively modulated osteoblast differentiation and resistance to stress-induced apoptosis. Taken together these studies have established a novel CaSR-Homer1-mTORC2-AKT signaling pathway in osteoblasts with functional consequences for cellular maturation and survival. In addition, we were able to establish reconstitute AKT-Ser$^{473}$ sensitivity to extracellular Ca$^{2+}$ in a non-osteoblast cell line. Thus, independent transfections of either Homer1 or CaSR into HEK-293 cells showed no AKT-Ser$^{473}$ sensitivity to extracellular Ca$^{2+}$, however co-transfection of Homer1 and CaSR established AKT-Ser$^{473}$ sensitivity to extracellular Ca$^{2+}$.

Discussion

Phosphorylation of AKT at Ser$^{473}$ is mediated by mTORC2 (9) and our earlier studies showed that activation of the CaSR induces AKT Ser$^{473}$ phosphorylation and nuclear β-catenin translocation in osteoblasts (6). The present study extends these findings to show that Homer1 acts as a previously unrecognized link between the CaSR, and mTORC2 upstream of AKT-Ser$^{473}$ (Figure 1). The proposal that Homer proteins, which primarily reside at the plasma membrane (21-23), promote AKT phosphorylation, is consistent with the recent finding that mTORC2 assembles at the plasma membrane prior to phosphorylating AKT at Ser$^{473}$ (13).

In this study we observed that in the mineralized bone of wt mice, areas exhibiting high levels of CaSR expression also exhibited high levels of Homer1 expression and confocal microscopy of primary human osteoblasts
demonstrated co-localization at the cellular level. Homer1 mediates Pi3K-L-dependent activation of PI3K upstream of AKT-Thr308 phosphorylation in hippocampal neurons (22) but a mechanism dependent on Homer1 and mTORC2 has not been described previously for the activation of AKT-Ser473. We observed that CaSR-mediated AKT-Ser473 phosphorylation, the resultant downstream phosphorylations of GSK3β and β-catenin, and the attendant nuclear translocation of β-catenin are all dependent on Homer1. These observations provide a molecular explanation for CaSR-stimulated stabilization of β-catenin in osteoblasts (6) (Figure 1). The requirement for CaSR and Homer1 to observe increased phosphorylation of both AKT-Ser473 and GSK3β-S9 in response to extracellular Ca2+ was also shown to be present in another commonly used osteoblast cell type, MG63 osteosarcoma cells.

It is not yet clear whether the interaction between the CaSR and Homer1 arises from a direct binding interaction or indirectly via an intermediate protein. In addition to the Class C GPCRs mGluR1 and mGluR5 (21-23,29), Homer1 interacts with the scaffolding protein SHANK1 as well as components of the actin cytoskeleton and cell signaling apparatus (27). For some of these interacting proteins, the Homer1-binding sequences have not yet been identified. A widely recognized consensus binding sequence PPxxF (25,40), which is present in the C-termini of both mGluR1 and mGluR5 is not present in the CaSR. However, some related sequences are present in the CaSR, including PTSF in intra-loop2 (residues 711-714; Uniprot P41180), and PENF in intra-loop3 (residues 799-802), which conform to a proposed variant of the consensus Homer1 binding sequence PxxF (22). Interestingly, the latter sequence is conserved in other members of GPCR family C including mGluR1 and mGluR5 (Uniprot Q13255 and P41594 respectively). Two other proteins that interacted with the Homer1-CaSR complex in the current study, mTOR and Rictor (Figure 2), also contain Homer binding sequences. mTOR contains the classical Homer1 binding sequence, PPKDF (residues 464-468, Uniprot P42345) and Rictor contains the variant sequence, PKGF (residues 812-815, Uniprot Q6R327).

We previously found that AKT stimulation in osteoblasts, downstream of the activated CaSR, promoted β-catenin stabilization and subsequent nuclear translocation that was dependent, in part, on inhibition of GSK3β activity via Ser9 phosphorylation, and that chemical inhibition of AKT by AKT-XI halted this process (6). We confirmed these findings in the present study and observed that the inhibitory phosphorylation sites of GSK3 were also suppressed by the mTOR kinase inhibitor Torin1 but not the PI3K inhibitor wortmannin (Figure Supp 2) – highlighting the relative importance of mTORC2- vs PI3K-dependent control of AKT.

Increases in the osteoblast differentiation marker ALP in response to extracellular Ca2+ were suppressed by silencing both CaSR and Homer1 in this study. It is of note that basal levels of ALP were suppressed by Homer1 silencing but not CaSR silencing. One interpretation of this finding is that Homer1 performs functions essential to cell differentiation, other than those that respond to increases in extracellular Ca2+. Clearly CaSR is essential to normal osteoblast differentiation (1,2), however the role of Homer1 is not known in bone cells. To further investigate, we performed a direct comparison between silencing CaSR and Homer1 on osteoblast viability in response to hydrogen peroxide-induced stress as measured by the viability dye Cell Titer Blue (Figure Supp 3). Silencing Homer1 reduced osteoblast viability, and the effect exceeded that observed in cells in which the CaSR had been silenced. The results of this experiment are consistent with the hypothesis that Homer1 is a key mediator of pro-survival pathways in bone cells and warrants further investigation.

Resistance to oxidative stress promotes bone quality in vivo (41). Reducing the level of either CaSR or Homer1 with siRNA significantly reduced the ability of elevated extracellular Ca2+ to support cell survival in the context of oxidative stress or sparse culture conditions. Hydrogen peroxide-induced apoptosis increased in response to Torin1, which inhibited mTORC2 and thus AKT-S473 phosphorylation but was unaffected by wortmannin which inhibits PI3K, supporting the idea that mTORC2-mediated phosphorylation of AKT, downstream of CaSR and Homer1, is a determinant of osteoblast survival.

The key observations of the present study indicate the existence of a receptor-scaffolding protein-intracellular protein kinase mechanism in support of cell survival and differentiation in Ca2+-
stimulated osteoblasts that requires the CaSR, Homer1, mTORC2, and AKT. To further explore whether these interactions might be generalizable to other cell types, we investigated whether it might be possible to reconstitute extracellular Ca\(^{2+}\)-stimulated AKT-Ser\(^{473}\) phosphorylation in HEK-293 cells. Consistent with our primary cell findings, Ca\(^{2+}\)-stimulated AKT-Ser\(^{473}\) phosphorylation was not observed in HEK-293 cells that had been stably transfectected with the CaSR or in HEK-293 cells that had been transiently transfectected with Homer1 alone. However, in HEK-CaSR cells that were transiently transfectected with Homer1c for the optimal period of 48 h we observed reconstitution of extracellular Ca\(^{2+}\)-stimulated AKT-Ser\(^{473}\) phosphorylation that exhibited an EC\(_{50}\) for Ca\(^{2+}\) of 1.0-2.0 mM and a maximal response at Ca\(^{2+}\)\(_{\text{m}}\) concentrations around 3-5 mM. HEK-293 cells have been reported previously to endogenously express both Rictor (42) and mTOR (42,43) and we used Western blot analysis to confirm this in the present study (data not shown). These observations, together with the observed impact of Homer1 knockdown in osteoblasts and osteosarcoma cells, indicate that expression of Homer1 in CaSR-expressing cells is required for the establishment of Ca\(^{2+}\)-stimulated AKT-Ser\(^{473}\) phosphorylation.

In conclusion, the present study demonstrates that in response to extracellular Ca\(^{2+}\), a novel protein complex, composed of the CaSR, Homer1 and mTORC2, forms in human osteoblasts and mediates a pathway that controls the phosphorylation of AKT leading to increased differentiation and survival (Figure 1). AKT phosphorylation supports an alternative pathway to the stabilization and nuclear translocation of β-catenin, a key driver of bone anabolism (15). This pathway may provide alternative targets for the development of new osteoporosis therapies. The findings demonstrate that in addition to roles in supporting neuromodulation and neuroplasticity by type-1 metabotropic glutamate receptors, Homer1 supports the control of bone cell survival and differentiation downstream of the CaSR, which monitors the levels of Ca\(^{2+}\) in the bone microenvironment.

**Experimental procedures**

**Materials**

All chemicals, including culture media were obtained from Sigma-Aldrich (MO, USA) unless otherwise specified. Fetal calf serum (FCS) and OptiMEM™ were from Life Technologies (CA, USA). All siRNA was from Santa Cruz Biotechnology. Protein A/G PLUS-agarose beads were from Santa Cruz Biotechnology. VeriBlot secondary antibodies were from Abcam (MA, USA). Inhibitors: Torin1 from Tocris (Bristol, UK), and wortmannin was from Sigma. The bicinchoninic acid (BCA) assay was obtained from Pierce (IL, USA). Caspase-3 Fluorogenic Substrate, N-acetyl-L-α-aspartyl-L-α-glutamyl-L-valyl-N-[2-oxo-4-(trifluoromethyl)]-2H-1-benzopyran-7-yl]-L-α-asparagine (Ac-DEVD-AFC) was from BD Pharmingen™ (CA, USA). Alkaline phosphatase (ALP) substrate 4-Nitrophenyl phosphate (4-pNP) was from Sigma. ALP (calf, intestinal) was from Promega (WI, USA). All absorbance and fluorescence measurements were carried out on a CLARIOstar microplate reader (BMG Labtech GmbH, Germany). Sonication of cell lysates was performed using a Branson Analog 250 cell disruptor with a double step (1/8 inch) microtip from Branson Ultrasonics (CT, USA).

**Methods**

**Cell culture-** Human osteoblasts were grown from the minced trabecular ends of fetal long bone in accordance with the National Health and Medical Research Council guidelines and with the approval of the University of Sydney Human Ethics Committee [approval number: 2012/043], as described previously (6,44-46). Each experiment was conducted using human osteoblasts from at least two different donors and cells were maintained routinely in DMEM containing 10% (v/v) FCS. These bone derived cells were moderately differentiated as previously described (6,44). Because the bone cells were from different donors some biological variations in responses were observed, as expected (6,44,45). Human Endocrine Kidney cells (HEK-293) (ATCC® CRL-1573; Virginia, USA) and MG-63 osteosarcoma cells (ATCC® CRL-1427) were maintained in DMEM containing 10% (v/v) FCS. The stably expressed wild-type human CaSR in HEK293 cells was the kind gift of Dr. Karen Krapcho and Dr. Edward Nemeth (NPS Pharmaceuticals).

**CaSR knockout mice-** Male and female mice with ablation of both alleles of Casr gene in C57/B6
background were generated by breeding the floxed-CaSR mice and the mice expressing Cre recombinase transgene under the control of a 2.3kb promoter of rat type I collagen α-subunit as described previously (1). All mice were housed in a pathogen-free climate-controlled room (22°C; 50% relative humidity) with a 12/12-hour dark/light cycle, given filtered water and standard chow containing 1.0% calcium and 0.7% phosphate, and euthanized at 3 months of age by isoflurane overdose for subsequent harvests of serum and bone samples. All animal procedures were approved by the Institutional Animal Care and Use Committee at the San Francisco Veterans Affairs Medical Center. Femurs and tibiae from wild type, heterozygous and homozygous osteoblast-specific CaSR knockout mice (1) were used for immunofluorescent (IF) and western blot analyses of either CaSR, Homer1 or osteoprotegerin (OPG). Femurs were snap frozen and kept at -80°C prior to protein extraction for western blot analysis. Tibiae were cleaned, fixed in 10% formalin, decalcified and sectioned at 8 microns thick for IF. Sections were stained for IF as described below.

**HEK-293 transient transfection** - Cells were transiently transfected using the X-tremeGENE HP DNA Transfection Reagent (Roche, Germany) under serum-free conditions and were used for subsequent experiments 48 h after transfection. The pRK5-Homer1c construct was the kind gift of Dr RaoZhu Lin and Prof Paul Worley (John Hopkins School of Medicine).

**siRNA transfection** - Each siRNA used in this study was a commercially supplied (Santa Cruz Biotech) pool of 3 target specific 19-25 nt siRNAs designed to knock down expression of the specific gene of interest. siRNA sequences are listed in Table Supp 1. siRNAs against all target transcripts were used at a concentration of 50 nM in the presence of siRNA transfection reagent (Santa Cruz Biotechnology) according to the manufacturer’s instructions. Control transfections containing a non-directed siRNA sequence were carried out simultaneously, on the same plate, for all experiments. Briefly, osteoblasts were transfected overnight under serum free conditions, and permitted to grow for an additional 24 h in complete growth media after transfection, which allowed efficient reduction to the level of targeted protein. Protein knockdown was confirmed by western blot.

**Immunofluorescence (mouse bones)** - Tibial sections were deparaffinised, cleared in xylene, rehydrated in ethanol steps and permeabilised in Triton X-100 (0.5%). Bone sections were then blocked in 2% bovine serum albumin for 30 min before being probed with anti-CaSR (goat polyclonal, clone F-19) or anti-Homer1 (rabbit polyclonal, clone H-174) followed by anti-goat Alexa Fluor 488 and anti-rabbit-Cy3. To visualize the nuclei, coverslips were mounted with UltraCruz™ Mounting Medium containing 4,6-diamidino-2-phenylindole (DAPI). Slides were observed under an Axio Imager 2 deconvolution microscope with Zeiss Apotome 2 imaging system (Zeiss, Oberkochen Germany) at the Advanced Microscopy Facility (Bosch Research Institute, University of Sydney).

**Immunofluorescence (human osteoblasts)** - Human osteoblasts were grown on poly-L-lysine coated coverslips and were fixed with 4% (w/v) paraformaldehyde followed by 100% ice-cold methanol and were processed with the following antibodies: anti-CaSR (Sigma Aldrich, mouse monoclonal, clone HL1499), anti-Homer1 (Santa Cruz, rabbit polyclonal, clone H-174) and isotype controls. Coverslips were then washed and incubated with anti-rabbit Alexa Fluor 488 (1:750; Santa Cruz) and anti-mouse Cy3 (1:750; Life Technologies) at room temperature for 60 min. To visualize the nuclei, coverslips were mounted with UltraCruz™ Mounting Medium containing DAPI. Slides were observed under a LSM510 Meta confocal laser microscope (Zeiss) at the Advanced Microscopy Facility (Bosch Research Institute, University of Sydney).

Images for colocalization analysis were taken using the 63x objective under oil. Background threshold levels were determined using ImageJ software (W.Rasband, NIH) and Pearson’s colocalization coefficients (47,48) were calculated using the Coloc2 plugin of ImageJ.

**Protein extraction from mouse bones** - Following the removal of bone marrow, total protein was extracted from mouse bones using a combination of de-mineralization and chemical denaturation as described previously (49). Protein extracts were precipitated from their chemical denaturation solutions and equal amounts of protein were subjected to western blot analysis, and densitometry was carried out on triplicate bands.
Western blot- For phosphoprotein detection, following inhibitor treatment or siRNA transfection cells were permitted to equilibrate for 90 minutes in OptiMEM™ (adjusted to contain a final concentration of 1 mM Ca\(^{2+}\)). The indicated concentration of Ca\(^{2+}\) (or the vehicle treatment) was then added to the cells for the indicated period, (typically 15 min) prior to lysis. Following treatment, monolayer cultures were solubilized and analysed by SDS-PAGE under reducing conditions followed by western blot to nitrocellulose membranes as previously described (6). Primary antibodies for phosphoprotein detection were all from Cell Signaling Technology (CST, MA, USA): AKT-S\(^{373}\) (mouse monoclonal, clone 587F11); AKT-S\(^{373}\) (rabbit monoclonal, clone D9E); AKT-T\(^{308}\) (mouse monoclonal, clone L32A4); GSK3α/β-S\(^{21/9}\) (rabbit polyclonal #9327); β-catenin-S\(^{552}\) (rabbit polyclonal #9566); mTOR-S\(^{2448}\) (rabbit polyclonal #2971). Total (pan) protein detection antibodies were: AKT (CST, mouse monoclonal, clone 40D4); GSK3β (CST, rabbit monoclonal, clone D5C5Z); β-catenin (Sigma Aldrich, rabbit polyclonal #C2206); mTOR (CST, rabbit polyclonal, #2972); β-actin (Santa Cruz Biotech, mouse monoclonal, clone C4); GAPDH (CST, rabbit monoclonal, clone 14C10); CaSR (Sigma Aldrich, mouse monoclonal, clone HL1499); Homer1 (Santa Cruz Biotech, mouse monoclonal, clone B5; rabbit polyclonal, #H-174); Gα12 (Santa Cruz Biotech, mouse monoclonal, clone E12); Raptor (CST, rabbit monoclonal, clone 24C12); Rictor (CST, rabbit monoclonal, clone D16H9); OPG (R&D Systems, biotinylated goat polyclonal #BAF805); LaminB1 (Thermo Fisher, mouse monoclonal, clone L5); GAPDH (CST, rabbit monoclonal, clone 14C10).

Alkaline phosphatase activity– Following treatment, human osteoblasts in triplicate wells of 96-well plates were lysed in PBS containing 0.1% (v/v) TX-100. An equal vol of 0.2 M glycine pH 9.5 containing 1 mg/mL 4-pNPP (ALP substrate; 4-Nitrophenyl phosphate) was added to start the enzymatic reaction. The reaction was simultaneously carried out with calf intestinal ALP (0-1 U) under the same conditions to construct a standard curve. Identically treated wells on the same plate were used for cell number correction determined by total cellular protein (BCA).

Caspase-3 activity- Following treatment, apoptosis was assessed in human osteoblasts in triplicate wells of 96-well plates via caspase-3 activity with the peptide substrate Ac-DEVD-AFC (BD Biosciences, NJ, USA) used according to the manufacturer’s instructions. Identically treated wells on the same plate were used for cell number correction determined by total cellular protein (BCA).

Nuclear localization of β-catenin– Detection of β-catenin localized to the nucleus was performed as previously described (6). Briefly, following treatment osteoblast monolayers were lysed in 10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM MgCl\(_2\), 0.5% (v/v) NP-40, with protease inhibitors for 5 min on ice. Lysates were centrifuged at 500 x g for 30 min at 4 °C, and the pellet washed 3 times with lysis buffer. Insoluble pellet was resuspended in a high salt buffer (10 mM Tris (pH 7.5), 0.4 M NaCl for 30 min on ice, centrifuged at 12,000 x g for 30 min at 4 °C, and the resulting supernatant represented the nuclear fraction. Protein concentrations were normalized between treatments (BCA), and equal amounts of protein were subjected to western blot analysis. LaminB1 was used as a loading control for the nuclear fraction.

Co-immunoprecipitation- Human osteoblasts in triplicate wells of 6-well plates were exposed to control (1.0 mM) or elevated (3.5 mM) Ca\(^{2+}\) concentration for 2 min, immunoprecipitated (IP) with anti-Homer-1-b/c using a variation of a protocol previously described (50). Briefly, following treatment the cell monolayer was lysed on ice in 50 mM Tris pH 7.5, 250 mM NaCl, 1% (v/v) NP-40 with protease inhibitors and lysates from triplicate wells were combined and processed through a 21G needle five times. Insoluble material was removed by centrifugation and the lysate was pre-cleared with protein-A/G PLUS agarose beads (20 µL/mL). Following protein concentration measurement, immunoprecipitation of 1 mg total cellular lysate with 1 µg/mL monoclonal anti-Homer1 (Santa Cruz (Biotech, rabbit polyclonal, #H-174) (or isotype control) was carried out overnight at 4°C with protein-A/G PLUS agarose (20 µL/mL) with orbital mixing. Beads were extensively washed in lysis buffer prior to detection of immunoprecipitated protein by western blot under reducing conditions using VeriBlot™ secondary antibodies (Abcam, MA, USA). As with other western blots, this experiment was performed 3 times for quantification.
**OPG protein** - OPG protein levels *in vitro* were detected by whole-cell ELISA. OPG protein levels in the supernatant of primary osteoblasts were routinely at the lower limit of detection by capture ELISA, but the protein was readily found to be associated with the cell monolayer. For this reason, a whole-cell ELISA was developed. Following treatment, cells were fixed with 10% neutral buffered formalin for 5 min and then permeabilised with three incubations with PBS/0.1% (v/v) TX-100 for 5 min each. Endogenous peroxidase was inactivated with 0.6% H$_2$O$_2$ in PBS for 15 min. Wells blocked with 5% (w/v) BSA in PBS for 30 min. Detection was with anti-OPG monoclonal antibody (mouse monoclonal, clone # 69127, R&D Systems, MN, USA), or an isotype control for background correction, in blocker at 4°C on rocking platform overnight followed by anti-Ig-HRP conjugated at 1 μg/mL in blocker for 1 h at RT and subsequent 3,3',5,5'-Tetramethylbenzidine detection. All steps were separated by five PBS/0.01% (v/v) Tween-20 washes.

**qPCR protocol for Wnt SuperArray** - We used the reverse transcription (RT$^2$) Profiler polymerase chain reaction (PCR) Array Mouse Wnt Signaling Pathway array purchased from SuperArray Bioscience (Frederick, MD). The complete gene list is available on [http://www.superarray.com](http://www.superarray.com). Total RNA from marrow flushed mouse bones was isolated using the RNA Stat-60 (Amsbio, Cambridge, MA, USA). First-strand cDNA synthesis reaction was performed as follows: 2 μg of extracted RNA was mixed with 10 μL of the SuperArray RT cocktail mix. The products were then incubated at 37°C for 1 hour and heated at 95°C for 5 minutes. Real-time-based SYBR green PCR was performed using a ViiA7 real-time PCR system (Thermo Fisher Scientific, USA) and the following thermal cycling condition was used: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Data analysis and the cycle threshold (CT) values, which were defined as the fractional cycle number at which the fluorescence passes an arbitrarily set threshold, were analyzed using the built-in ViiA7 analytic software. The CT value of each gene was normalized to that of β-actin, which is included in this commercially available kit.

**Cell Titer Blue** - The cell viability dye Cell Titer Blue (Promega) was used according to the manufacturer’s instruction. Fluorescence of the dye was measured at λEx:560/λEm:590.

**Statistics and data analysis** - Experiments were performed in triplicate. Each experiment was repeated at least three times, with cells from different donors, and the data are reported as means ± standard deviation. One-way analysis of variance with the Tukey post-test were used to determine significant differences between treatments using Prism (GraphPad Software Inc).
Acknowledgements: No acknowledgements.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

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FOOTNOTES
Funding was provided by the National Health and Medical Research Council of Australia (Grant #: 1008282).

The abbreviations used are: CaSR, calcium-sensing receptor; AKT, acutely transforming retrovirus AKT8 in rodent T-cell lymphoma; GPCR, G-Protein Coupled Receptor; mGluR, metabotropic glutamate receptor; CNS, central nervous system; PDK1, 3-phosphoinositide-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase (PI3K); mTOR, mechanistic target of Rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; Rictor, rapamycin-insensitive companion of mTOR; Raptor, regulatory associated protein of mTOR; LRP5/6, LDL receptor-related protein-5&6; GSK3, glycogen synthase kinase-3; ALP, alkaline phosphatase; ERK, extracellular signal-regulated kinase; OPG, osteoprotegerin.
Table 1: Quantitative RT-PCR microarray analysis of the impact of CaSR depletion on the expression of various genes of the canonical Wnt pathway. Total RNA was extracted from marrow-flushed 2.3Col(I)-CaSR and wt bone and analysed with the Wnt Signaling Pathway array kit (SuperArray Bioscience). p value was generated by t-test.

| Gene     | \(\Delta\Delta Ct\) | Expression fold change \((2^{\Delta\Delta Ct})\) CaSR\((-/-) / wt\) | p value |
|----------|----------------------|---------------------------------------------------------------|---------|
| Axin1    | 0.56                 | 0.68                                                          | 0.032   |
| Ccnd3    | 1.02                 | 0.49                                                          | 0.017   |
| Csnk2a1  | 0.71                 | 0.61                                                          | 0.011   |
| Ctnnb1   | 0.36                 | 0.78                                                          | 0.019   |
| Daam1    | 0.96                 | 0.52                                                          | 0.011   |
| Dvl2     | 0.43                 | 0.74                                                          | 0.042   |
| Jun      | 0.64                 | 0.64                                                          | 0.001   |
| Kremen1  | 0.22                 | 0.86                                                          | 0.028   |
| Lef1     | 0.89                 | 0.54                                                          | 0.011   |
| Lrp5     | 1.04                 | 0.49                                                          | 0.012   |
| Nkd1     | 1.20                 | 0.44                                                          | 0.004   |
| Tcf7     | 0.49                 | 0.71                                                          | 0.043   |
| Wif1     | 0.97                 | 0.51                                                          | 0.033   |
| Wisp1    | 1.00                 | 0.50                                                          | 0.005   |
| Wnt1     | 2.10                 | 0.23                                                          | 0.002   |
| Wnt10a   | 1.20                 | 0.44                                                          | 0.004   |
| Actb (control) | 0.02 | 0.99 | 0.52 |
| Gusb (control) | -0.6 | 1.52 | 0.076 |
**Figure legends**

**Figure 1** – We propose Homer1 may link CaSR to mTORC2 in osteoblasts to promote AKT dependent β-catenin stabilization. Proposal of current study is that Homer-1 may link CaSR to mTORC2 activation upstream of AKT (indicated by “?”). AKT phosphorylation is known to occur via two discrete mechanisms; PI3K-dependent phosphorylation at Thr<sup>308</sup>, and mTORC2-dependent phosphorylation at Ser<sup>473</sup>. AKT subsequently inhibits GSK3 and directly phosphorylates β-catenin to promote β-catenin stabilization and allowing subsequent nuclear translocation leading to cellular differentiation. AKT also suppresses apoptosis and promotes growth in the cell. Kinase target of Torin1 is mTOR. PM = plasma membrane.

**Figure 2** – CaSR and Homer1 were necessary for mTORC2-dependent dual phosphorylation of AKT in response to elevated extracellular Ca<sup>2+</sup> in osteoblasts. (A) Phosphorylations of AKT at Thr<sup>308</sup>/Ser<sup>473</sup>, from same osteoblasts by western blot in response to Ca<sup>2+</sup> shift 1-2 mM over 1h. (B) Western blot of AKT phosphorylated at Ser<sup>473</sup>/Thr<sup>308</sup> and mTOR phosphorylated at Ser<sup>2448</sup> in response to Ca<sup>2+</sup> shift 1-2 mM for 15 min in the presence of Torin1 (pre-treatment 5 min). Concentration shown as fold times the half maximal inhibitory concentration (IC<sub>50</sub>). IC<sub>50</sub> Torin1 ~ 2 nM. (C&E) Western blot of phosphorylations of AKT at Thr<sup>308</sup>/Ser<sup>473</sup>, in response to Ca<sup>2+</sup> shift 1-2 mM for 15 min after (C) CaSR (siCaSR) or (E) Homer1 (siHomer1) protein levels reduced (“+”) compared to control transfected cells (“-”). (D&F) Densitometry of triplicate blots shown in for silenced (C) CaSR and (E) Homer1 respectively. # Solid, vertical black lines on the presented western blot indicate where lanes have been spliced from the same western blot for presentation purposes. The data shown in C-F are also presented in Figure 4 as part of the broader signaling cascade investigated in the study.

**Figure 3** - CaSR and Homer1 were co-expressed in mineralized bone, co-localized in vitro, and co-immunoprecipitated with mTOR and the mTORC2-specific protein Rictor. (A) Deconvolution microscopy images of the mineralized area of the long bones of wt (top row) or CaSR-/-(bottom row) mice stained for CaSR (green) or Homer1 (red). Nuclei (blue) stained with DAPI. Scale=50μm. (B) Confocal microscopy images of a monolayer culture of primary human osteoblasts taken with 10x objective (top row, scale bar = 10μm) or a single osteoblast taken under oil with 63x objective (bottom row, scale bar = 2μm) stained for CaSR (green) or Homer (red). Nuclei (blue) stained with DAPI. (C) Human osteoblasts were exposed to Ca<sup>2+</sup> shift 1-3.5 mM for 2 min then lysed with a non-ionic detergent. Soluble protein was immunoprecipitated with anti-Homer1 (H) or an isotype control (Iso). Detection of (i) Homer1, as a control between Ca<sup>2+</sup> treatments, (ii) CaSR, (iii) mTOR, (iv) Rictor, and (v) Raptor by western blot from the immunoprecipitated fraction. “total lysate” is western blot of non-immunoprecipitated osteoblast lysate; “#” non-glycosylated CaSR (D) Densitometry from triplicate blots, where bands were detected, shown in “C” for (i) Homer1, (ii) CaSR, (iii) mTOR, and (iv) Rictor. ns no significance, * p<0.05, ***p<0.001.

**Figure 4** - CaSR and Homer1 modulated Ca<sup>2+</sup>-dependent activation of the AKT pathway in human osteoblasts. (A-D) Phosphorylation of AKT at Ser<sup>473</sup>/Thr<sup>308</sup>, GSK3α-Ser<sup>21</sup>, GSK3β-Ser<sup>9</sup>, β-catenin-Ser<sup>552</sup>, mTOR-Ser<sup>2448</sup> and β-actin (loading control) from human osteoblasts by western blot. (A) Osteoblasts exposed to various Ca<sup>2+</sup> concentrations for 15 min. (B-D i) Osteoblasts were exposed to Ca<sup>2+</sup> shift from 1-2 mM for 15 min after silencing: (B) CaSR (siCaSR); (C) Homer1 (siHom1); (D) Gα<sub>12</sub> (siGα<sub>12</sub>) (“+”). and the lysates were then processed for western blot detection of various proteins and phosphoproteins as shown. The impact of siRNA on the silenced gene is shown at the bottom of each column in each of B(i), C(i) and D(i) where expression shown as “silenced gene”. For each transfection, the effect of a non-directed control siRNA sequence (siCTRL) is shown (“-”). (B-C ii) Densitometry results from triplicate blots shown in B(i), C(i) and D(i). *p<0.05, **p<0.01, ns not sig diff. # Solid, vertical black lines on the presented western blot indicate where lanes have been spliced from the same western blot for presentation purposes. The AKT, CaSR and Homer1 data shown in B-C are also shown in Figure 2 which focuses independently on AKT.
Figure 5: CaSR and Homer1 modulated Ca$^{2+}$-dependent β-catenin nuclear translocation in human osteoblasts. (A) Nuclear β-catenin is shown in response to (i) Ca$^{2+}$ shift from 1 to 2 mM over a 60 min period, or (ii, iii) a 30 min Ca$^{2+}$ shift 1 to 2 mM with reduction in CaSR protein level (siCaSR; ii) or reduction in Homer1 protein level (siHomer1; iii) by siRNA. The nuclear protein Lamin B1 was used as a loading control for the nuclear fraction. (iv-v) Densitometry from triplicate blots for silenced CaSR and Homer1. (vi) OPG protein levels, corrected for total cell protein, measured by whole-cell ELISA in cells that were incubated for 24 h in 2 mM Ca$^{2+}$ ± AKT kinase inhibitor AKT-XI (20 μM). (B) Western blot of OPG that was chemically extracted from marrow flushed mouse long bones from wt (CaSR+/+), heterozygous (CaSR+-) or CaSR knockout (CaSR-/-). β-actin loading control. * p<0.05, ** p<0.01, *** p<0.001 compared to vehicle.

Figure 6: CaSR and Homer1 modulated Ca$^{2+}$-dependent phosphorylation of AKT-Ser$^{473}$ and GSK3β-Ser$^{9}$ in MG63 osteosarcoma cells. (A) Western blot showing level of AKT-Ser$^{473}$/total AKT (pan), GSK3β-Ser$^{9}$/total GSK3β (pan) and α-tubulin (loading control) in response to various Ca$^{2+}$ concentrations for 15 min in MG63 osteosarcoma cells. Prior to Ca$^{2+}$ treatment, cells were transfected with siRNA directed at Homer1 (siHom), CaSR (siCaR) or a non-directed sequence (siCTRL) for 48 h. Densitometry values (dens) were calculated by dividing the detected level of phosphorylated protein by the level of total protein (Image J), and normalizing this number to the ratio obtained from the 1 mM Ca$^{2+}$ lane for each silenced condition. (B) Western blot showing expression level of Homer1 and CaSR in response to each directed siRNA (+) compared to cells transfected with a non-directed sequence (-) in MG63 cells. # Solid, vertical black lines on the presented western blot (B panel, siCaR blots only) indicate where lanes have been spliced from the same western blot for presentation purposes. The experiment was independently repeated with similar results.

Figure 7 – Ca$^{2+}$-dependent stimulation of alkaline phosphatase activity and rescue from oxidative stress-induced apoptosis depend on Homer1 and CaSR. ALP activity in response to various Ca$^{2+}$ concentrations for 3 d in (A) absence of siRNA, (B) presence of siRNA to CaSR (siCaSR), (C) presence of siRNA to Homer1 (siHomer1). For each transfection, the effect of a non-directed control siRNA sequence (siCTRL) is shown. ALP corrected for total cellular protein (t.c.p). (D-E) Caspase-3 activity in (D) osteoblasts treated with Ca$^{2+}$ at indicated concentrations for 5h then 125μM H$_2$O$_2$ for 2.5h. (E-F) Osteoblasts transfected with siRNA to reduce (E) CaSR (siCaSR) or (F) Homer1 (siHomer1) or a non-directed control sequence (siCTRL) and then treated with 2mM Ca$^{2+}$, or vehicle (1 mM Ca$^{2+}$) for 5h then 125μM H$_2$O$_2$ for 2.5h. (G-H) Caspase-3 in osteoblasts treated with 2mM Ca$^{2+}$ and (G) Torin1 (IC$_{50}$=2 nM) or (H) wortmannin (IC$_{50}$=1nM) for 5 h then 125 μM H$_2$O$_2$ for 2.5h. Caspase-3 corrected for t.c.p. ns not significant, *p<0.05, **p<0.01, ***p<0.001 compared to vehicle.

Figure 8: Co-transfection of HEK-293 cells with CaSR and Homer1c established AKT-Ser$^{473}$ sensitivity to extracellular Ca$^{2+}$. (A) Western blot of HEK-293 cells transiently transfected with Homer1c (HEK-Homer1c) or non-transfected cells (NT) as a control, showing Homer1c protein levels at various time points post transfection. 48 h post-transfection was chosen as the time point for subsequent signaling analyses (B-D) Western blot of AKT-Ser$^{473}$ phosphorylation levels in response to 0.1-5 mM extracellular Ca$^{2+}$ in (B) HEK-CaSR, (C) HEK-293 cells transiently transfected with Homer1c, or (D) HEK-CaSR cells transiently transfected with Homer1c. ns, not sig diff; ** p<0.01, ***p<0.001 compared to 0.1 mM Ca$^{2+}$; ## p<0.01 compared to 0.5 mM Ca$^{2+}$; $ p<0.05 compared to 1 mM Ca$^{2+}$.
Homer1 links CaSR to mTORC2

Figure 1

CaSR

PM

Ca\(^{2+}\)

Homer-1

?↓

Torin1

mTORC2

PI3K

PDK1

AKT

GSK3\(\text{S}\,573\)

\(\text{S}\,219\)\(\mid\)\(\text{S}\,552\)

\(\text{S}\,2448\)

\(\beta\)-catenin

caspase-3

mTORC1

“differentiation, growth & survival”
Figure 2
Figure 3

Homer1 links CaSR to mTORC2

A (Mineralized mouse bone)

B (Primary human osteoblasts)

C (Immunoprecipitation)

D (Immunoprecipitation densitometry)
Figure 4

Homer1 links CaSR to mTORC2
Figure 5

A

(i) time after Ca^{2+}_i \rightarrow 2 \text{ mM (min)}

| kDa | 0 | 15 | 30 | 45 | 60 | - | + | - | + |
|-----|---|----|----|----|----|---|---|---|---|

(ii) siCaSR

(iii) siHomer1

|       | \(\beta\)-cat(nuc) | lamB1(nuc) |
|-------|---------------------|------------|
|       | 120                 | 85         |
|       | 85                  | 50         |

(iv) si-CaSR

(v) si-Homer

(vi) OPG

B

CaSR genotype

| (+/+) | (+/-) | (-/-) |
|-------|-------|-------|

| CaSR genotype | OPG | \(\beta\)-act |
|---------------|-----|---------------|
| (+/+)         |     |               |
| (+/-)         |     |               |
| (-/-)         |     |               |
Homer1 links CaSR to mTORC2

Figure 6

A

| siCTRL | siHom | siCaR |
|--------|-------|-------|
| [Ca^{2+}]_0 (mM) |
| 1 | 1.5 | 3 | 1 | 1.5 | 3 | 1 | 1.5 | 3 |
| pAKT-S473 |
| AKT (pan) |
| pGSK3β-S9 |
| GSK3β (pan) |
| α-tubulin |

B

| kDa | Homer1 | α-tubulin |
|-----|--------|-----------|
| 52  |        |           |
| 37  |        |           |
| 66  |        |           |
| 52  |        |           |

| kDa | CaSR | α-tubulin |
|-----|------|-----------|
| 175 |      |           |
| 130 |      |           |
| 66  |      |           |
| 52  |      |           |
Figure 8

A
HEK-Homer1c; Homer1c expression

| kDa | NT | 12 | 24 | 36 | 48 |
|-----|----|----|----|----|----|
| 50  |    |    |    |    |    |
| 40  |    |    |    |    |    |
| 30  |    |    |    |    |    |
| 25  |    |    |    |    |    |

B
HEK-CaSR; Ca\textsuperscript{2+}-dependent pAKT-Ser\textsuperscript{473}

| [Ca\textsuperscript{2+}] (mM) | 0.1 | 0.5 | 1.0 | 2.0 | 3.0 | 5.0 |
|------------------------------|-----|-----|-----|-----|-----|-----|
| kDa                         | 85  | 60  | 40  | 25  |     |     |

C
HEK-Homer1c; Ca\textsuperscript{2+}-dependent pAKT-Ser\textsuperscript{473}

D
HEK-CaSR/Homer1c; Ca\textsuperscript{2+}-dependent pAKT-Ser\textsuperscript{473}
**Homer1 mediates CaSR-dependent activation of mTOR complex 2 and initiates a novel pathway for AKT-dependent β-catenin stabilization in osteoblasts**

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*J. Biol. Chem.* published online September 16, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA118.006587

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