TITLE: The mTORC2 regulator Homer1 modulates protein levels and sub-cellular localization of the CaSR in human osteoblasts.

RUNNING TITLE: Homer1 controls CaSR protein in osteoblasts.

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ABSTRACT

We recently found that in human osteoblasts Homer1 complexes to CaSR and mediates AKT initiation via mTORC2 leading to beneficial effects in osteoblasts including β-catenin stabilization and mTORC1 activation (doi: 10.1074/jbc.RA118.006587). Herein we further investigated the relationship between Homer1 and CaSR and demonstrate a link between the protein levels of CaSR and Homer1 in human osteoblasts in primary culture. Thus, when siRNA was used to suppress the CaSR, we observed upregulated Homer1 levels and when siRNA was used to suppress Homer1 we observed downregulated CaSR protein levels using immunofluorescence staining of cultured osteoblasts as well as western blot analyses of cell protein extracts. This finding was confirmed *in vivo* as the bone cells from osteoblast specific CaSR(-/-) mice showed increased Homer1 expression compared to wild-type. Furthermore, when the commonly used osteosarcoma cell lines MG63 and SAOS-2 were compared to primary osteoblasts, higher levels of Homer1 protein were associated with increased protein levels of the CaSR as well as mTOR and Rictor. CaSR and Homer1 protein were both expressed in osteocytes embedded in the long bones of wild-type mice, and immunofluorescent studies of these cells revealed that Homer1 protein sub-cellular localization was markedly altered in the osteocytes of CaSR(-/-) mice compared to wt. The study identifies additional roles for Homer1 in the control of the protein level and subcellular localization of CaSR in cells of the osteoblast lineage, in addition to its established role of mTORC2 activation downstream of the receptor.

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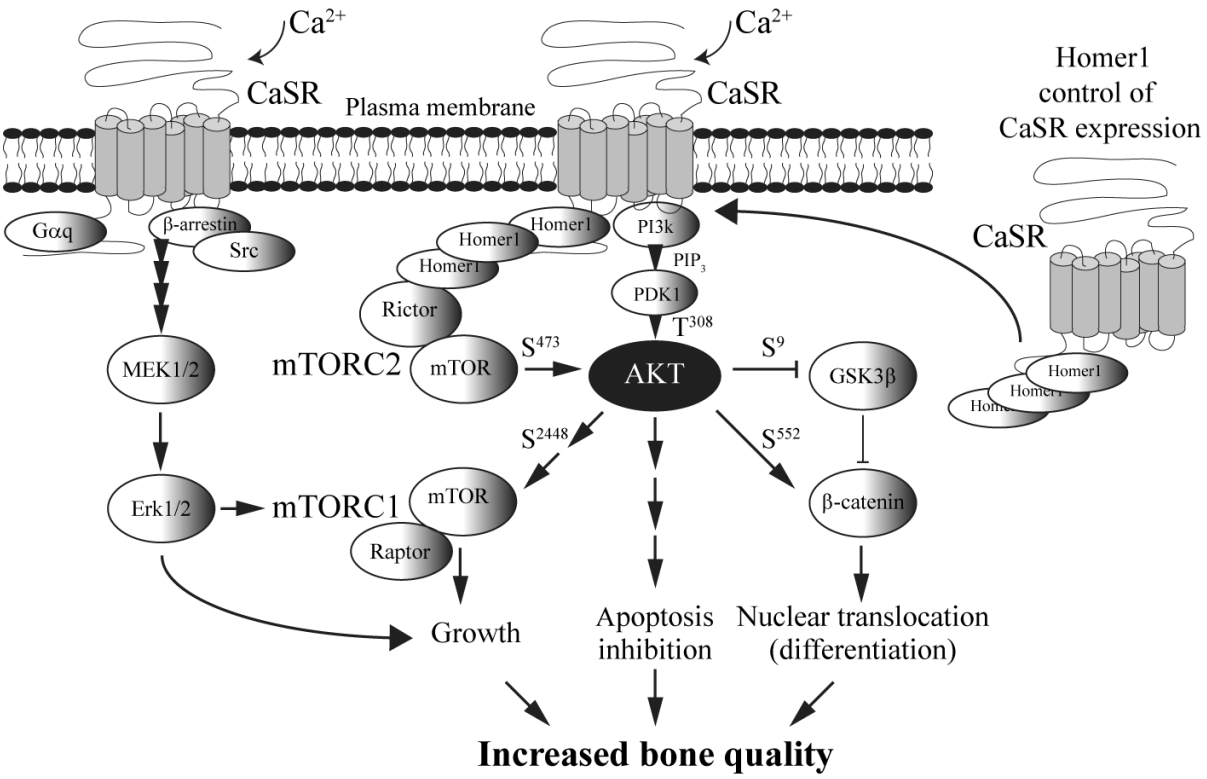
GRAPHICAL ABSTRACT



GRAPHICAL ABSTRACT- Homer1 exerts dual control over CaSR expression and Ca^{2+}-dependent AKT signaling in human osteoblasts. Homer1 complexed to mTORC2 to control Ca^{2+}/CaSR-dependent AKT signaling in osteoblasts (1). Following an increase in the concentration of Ca^{2+}, the native ligand of the CaSR, the interaction of Homer1, CaSR and the mTORC2-specific protein Rictor increased acutely in osteoblasts. Arising from the formation of this Homer1-dependent complex, Ca^{2+}-induced AKT signaling was observed, resulting in increased β-catenin translocation to the nucleus, mTORC1 activation, and enhanced differentiation and protection from stress-induced apoptosis of osteoblasts (1). Ca^{2+}-dependent $ERK_{1/2}$ activation occurs via a distinct pathway that has previously been shown to be modulated via $G\alpha_q$ proteins and the β-arrestin pathway. Herein we also show that Homer1 and CaSR protein levels are interdependent both in vitro and in vivo.

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INTRODUCTION

In bone, the roles of canonical Wnts (e.g., Wnt3a) in activating LRP5/6 and Frizzled-dependent osteoblastogenesis via β-catenin activation and transfer to the cell nucleus is well-established (2). The roles of several key regulators have been identified including the osteocyte-derived inhibitor sclerostin, whose levels fall in response to mechanical stimuli (3). However, the existence and identity of nutrient-dependent signaling pathways that lead to β-catenin stabilization and contribute to this signaling pathway, are largely unknown.

We previously demonstrated that activation of the nutrient-sensing class C GPCR, the CaSR, by Sr^{2+} in human osteoblasts promotes a novel Akt-dependent signaling pathway upstream of Wnt and its canonical mediator of osteoblastogenesis, β-catenin (4). More recently, while investigating the mechanism that supports this pathway, we demonstrated that the CaSR also mediates osteoblastogenesis in response to the key physiologically regulated divalent cation Ca^{2+} and uncovered roles for Homer1 and mTOR complex-2 (mTORC2) downstream of the Ca^{2+}-stimulated CaSR and upstream of Akt (1).

The importance of CaSR in bone is well established (5-7). CaSR is a class C G-protein–coupled receptor (GPCR) that is closely related to the metabotropic glutamate receptors (mGluRs). Homer proteins are known to act as scaffolds for the mGluR proteins, promoting the assembly of signaling complexes permitting the activation of growth and survival pathways such as AKT and ERK (8-10). We have recently shown that Homer also directly interacts with CaSR, and mTORC2 in osteoblasts resulting in AKT phosphorylation (1). Given the direct interaction of Homer and CaSR proteins, we have further predicted that Homer proteins may exert chaperone-like function on CaSR thereby promoting stability of CaSR in the cell.

In the current study we set out to determine whether Homer1 stabilizes and promotes CaSR protein levels in bone cells. We have assessed whether CaSR and Homer1 might impact each other's expression at the protein level using siRNA to suppress separately CaSR and Homer1 in human

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osteoblasts in primary culture, compared Homer1 expression levels in conditional osteoblast specific CaSR null mice, and extended the analyses to include osteocytes as well as osteoblasts.

RESULTS

Protein levels of Homer1 and CaSR were linked in primary human osteoblasts.

Homer1 and CaSR proteins were detected in cultured primary human osteoblasts by immunofluorescence (Figure 1A i-iv). Since Homer1 modulates the expression of several other known binding proteins (11,12), we tested whether reducing Homer1 protein affected CaSR protein levels. Confocal microscopy showed that knockdown of Homer1 by siRNA reduced Homer1 (Figure 1A vii vs iii), and also reduced CaSR protein levels compared to control transfected cells (Figure 1A vi vs 1A ii). Conversely, siRNA-mediated knockdown of the CaSR, whilst reducing CaSR protein levels as expected (Figure 1A x) *increased* protein levels of Homer1 (Figure 1A xi vs 1A iii).

Western blot analysis was used to confirm the relationship between Homer1 and CaSR proteins shown by immunofluorescence in Figure 1A (Figure 1B). Western blot was in agreement with the immunofluorescence data, where siRNA directed at Homer1 decreased Homer1 protein and CaSR protein compared to control transfected cells. siRNA directed at CaSR significantly reduced CaSR protein but resulted in *higher* Homer1 protein levels (Figure 1B).

Homer1 protein expression was increased in the long bones of osteoblast-specific CaSR knockout mice.

Confocal microscopy was used to detect protein levels of Homer1 and CaSR in osteocytes (Figure 2A) and osteoblasts lining the surface of long bones (Figure 2B) of wild-type (CaSR+/+) or osteoblast specific CaSR null (CaSR-/-) mice (5). As expected, no CaSR protein was detected in osteoblast-lineage cells from CaSR null mice. To quantify Homer1 protein levels from the various CaSR genotypes, total protein was chemically extracted from the long bones of CaSR+/+, CaSR +/- and

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CaSR-/- animals, and then subjected to western blot analysis (Figure 2C). Homer1 was significantly higher in CaSR-/- mice compared to CaSR+/+ (Figure 2C, 2D $p<0.01$). In CaSR+/- mice there was a non-significant increase in Homer1 when compared to CaSR+/+ (Figure 2C, D). As expected, CaSR, as a membrane protein, was not readily solubilized with guanidine based chemical extraction of total bone, though the CaSR was clearly detected in wt (Figure 2C) and overall the findings were visually consistent with those of the IF analyses (Figure 2A, 2B). Taken together these *in vitro* and *in vivo* findings indicate that the protein levels of CaSR and Homer1 are interdependent.

CaSR modulated the sub-cellular localization of Homer1 in osteoblast-lineage cells in vivo.

We have previously shown that CaSR and Homer1 proteins co-immunoprecipitate *in vitro* (1). The confocal microscope images shown in Figure 2A show that while wild-type osteocytes exhibited a very similar cytoplasmic distribution of both CaSR and Homer1, the CaSR-/- phenotype resulted in Homer1 staining that localized to and around the nucleus - which possibly represented accumulation of the protein in the ER/Golgi (Figure 2E). Multiple images of osteocytes from the long bones of both CaSR+/+ and CaSR-/- mice, shown in Figure 2E, indicate the change in Homer1 cellular localization described above was also consistently observed in osteocytes between CaSR wt and null phenotypes (Figure 2E, white arrows), and was also observed in bone lining cells (Figure 2B, white arrows).

A correlation existed between the native protein levels of CaSR and Homer1 in the osteosarcoma cell lines MG63 and SAOS-2.

To examine the relationship between CaSR and Homer1 protein levels in osteoblasts we examined the native expression in two commonly used osteosarcoma cell lines – MG63 (ATCC® CRL-1427™) and SAOS-2 (ATCC® HTB-85™) and compared these levels to primary human osteoblasts used in the above analyses. Both osteosarcoma cell lines exhibited markedly enhanced Homer1 expression compared to primary osteoblasts, along with increased expression of CaSR, mTOR, the mTORC2 specific protein Rictor, but not the mTORC1 specific Raptor and the effects were particularly pronounced in SAOS-2 (Figure 3). Whether increased CaSR/Homer1 expression and resultant AKT

activation contributes to the high survival, high growth phenotype of osteosarcoma cells and are thus potential targets for the chemotherapy of osteosarcoma, remains to be determined.

DISCUSSION

Given the importance of the CaSR in the development of bone phenotype and for mediating the positive effects of dietary calcium on bone (5-7,13), the observations that Homer1 promotes CaSR protein levels and is critical for CaSR function in osteoblasts points to the existence of previously unknown roles for Homer1 in skeletal development and maintenance, and possibly in other tissues as CaSR is ubiquitously expressed. Although this study focused on the long form of Homer1 (commonly termed the canonical sequence “Homer1b” or “Homer1c”; Uniprot identifier Q86YM7-1), we also detected the short form of Homer1, Homer-1a (Q86YM7-3), and other protein expression of the closely related Homer2 (Q9NSB8) and Homer3 (Q9NSC5) in primary human osteoblasts and the protein levels detected were consistent across primary cell donors (Figure Supp 1). Given the high degree of homology between these proteins it is possible that functional crossover, or redundancy of function, does occur. Importantly this may explain the lack of a reported bone phenotype in Homer1 knockout mice (14-16). In support of this argument, we observed that Homer2 protein levels in bone were also altered by CaSR knockout in mice, compared to wild-type, and importantly this change in protein level was in the opposite direction to Homer1, suggesting a compensation mechanism may exist in osteoblasts between various Homer isoforms (Supplemental Figure 2). Therefore, it is plausible to suggest that the expression level of other Homer isoforms could be modulated in bones of Homer1 k/o mice to provide compensatory function for the loss of Homer1. Although no bone phenotype has been reported in the mouse model, importantly it has been shown that the *Homer1* gene is linked to positive regulation of bone mass in postmenopausal women(17).

When CaSR protein was reduced in cells via siRNA or in CaSR-/- mice, we observed a pronounced increase in levels of Homer1 protein, consistent with the proposal that full normal CaSR function and expression requires Homer1. We propose that the increased expression of Homer1 in response to

reduced CaSR protein is a compensatory mechanism of the cell. In addition to the high degree of co-localization of Homer1 and CaSR in bone cells (1), an analysis of Homer1 protein distribution in CaSR-/- osteocytes and bone surface lining cells of mice showed that localization was clearly altered in a manner consistent with its accumulation in the ER in the absence of the CaSR. Retention of Homer1 in the ER, has also been reported in the absence of mGluR1 or 5 (18), and likely represents a shift in Homer1 cellular distribution in response to the absence of a native binding protein.

The findings in the present study suggest that the protein levels of CaSR and Homer1 are co-regulated. From the available data we are unable to determine whether the co-modulation of Homer1 and CaSR is due to protein stability or transcript expression – however a clear relationship exists at the protein level which is the critical point for assessing a role for the two proteins in cellular function. Within this study we did also look at mRNA levels in primary human osteoblasts in response to siRNA directed at both Homer1 and CaSR. Unfortunately, we did not observe consistency across osteoblasts donors with these analyses, and therefore we did not present mRNA data in the current study. Nevertheless, we propose that ultimately the important finding here is at the protein level, and potential transcription effects are not essential to the study conclusions.

To our knowledge, this is the first example of an increase in Homer1 arising from decreased levels of one of its binding proteins. The observation that CaSR protein levels depend in part, on Homer1 has also not been previously reported. Although the mechanism by which the normal presence of Homer1 increases CaSR protein levels is unclear, it seems plausible that in the process of forming stable protein complexes, Homer1 stabilizes the total cellular pool of CaSR protein and slows the rate at which it is directed to lysosomes for degradation (19). Previously, over-expression of Homer1b was reported to perturb mGluR5 protein trafficking and/or expression with different effects in different cell types (20,21).

We previously found that CaSR-dependent AKT stimulation in human osteoblasts promoted β-catenin stabilization and subsequent nuclear translocation that was dependent, in part, on inhibition of GSK3β

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activity via Ser^9 phosphorylation, and that direct chemical inhibition of AKT halted this process (4). Furthermore, we recently showed that this AKT-dependent process required Homer1, which links CaSR to mTORC2 – the point of AKT initiation (1). The findings of the present study show that, in addition to a direct effect on mTORC2/AKT signaling, Homer1 also regulates CaSR protein level in bone cells.

In conclusion, here we show that the long form of Homer1 positively regulated CaSR protein levels in primary human osteoblasts. Furthermore, in the absence of CaSR, Homer1 protein levels were increased and its sub-cellular localization was clearly altered. These effects are reported using both in vitro and in vivo models. The findings support the hypothesis that in addition to roles in supporting neuromodulation and neuroplasticity by type-1 metabotropic glutamate receptors, Homer1 maintains the protein level and AKT signaling capacity (1) of another nutrient-sensing GPCR of the same family, CaSR. The study supports the hypothesis that Homer proteins play more general roles outside the CNS in controlling the activity of family C GPCRs.

MATERIALS AND METHODS

All chemicals, including culture media were obtained from Sigma-Aldrich (MO, USA) unless otherwise specified.

Cell culture- The study was conducted using human osteoblasts from several different donors. 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. 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: 01/02/40), as described previously (22). These bone derived cells were moderately differentiated as previously described (4). Because the bone cells were from different donors some biological variations in responses were observed, as expected (4,23,24). SAOS-2 and MG63 cell lines, were maintained under similar conditions.

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CaSR knockout mice- Femurs and tibiae from wild type, heterozygous and homozygous osteoblast-specific CaSR knockout mice (5) were used for immunofluorescent (IF) and western blot analyses of CaSR and Homer isoforms. Femurs were snap frozen and kept at -80^{o}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.

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, Santa Cruz Biotechnology clone F-19) or anti-Homer-1b/c (rabbit polyclonal, Santa Cruz Biotechnology clone H-174) followed by anti-goat Alexa Fluor 488 (1:750; Santa Cruz Biotechnology) and anti-rabbit-Cy3 (1:750; Life Technologies). To visualize the nuclei, coverslips were mounted with UltraCruz™ Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology).

Immunofluorescence (human osteoblasts) – Human osteoblasts were grown on poly-L-lysine coated coverslips. At ~60% confluence, cells were transfected with the indicated siRNA as described below. Human osteoblasts were fixed with 4% (w/v) paraformaldehyde followed by 100% ice-cold methanol and were processed with the following antibodies: anti-CaSR (mouse monoclonal, Sigma clone HL1499), anti-Homer-1b/c (rabbit) 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.

siRNA transfection - siRNAs against all target transcripts (Homer1: sc-35581; CaSR: sc-44373 both from Santa Cruz Biotechnology) 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.

Chemical denaturation of whole mouse bones followed by protein level measurement by western blot

Total protein was chemically extracted from mouse long bones as previously described (1).

Western blot analyses - Human osteoblasts in triplicate wells of 6-well plates were exposed to control or elevated Ca^{2+} in the absence or presence of inhibitors or following siRNA transfection as described above. Monolayer cultures were solubilized and analysed by SDS-PAGE under reducing conditions followed by western blot to nitrocellulose membranes as previously described (4).

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).

Data availability- Requests for data and materials collected from the study should be made to the corresponding author.

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CONFLICTS OF INTEREST

The authors have no conflicts.

CONTRIBUTIONS: Study design: MSR, AC and RSM. Study conduct: MSR. Data Collection: MSR, TCBS, DM, WC and ZC. Data analysis: MSR. Data interpretation: MSR, AC and RSM.

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Drafting manuscript: MSR, AC and RSM. Revising manuscript content: MSR, WC, AC and RSM. Approving final version of manuscript: MSR , TCBS, AC and RSM. MSR takes responsibility for the integrity for the data analysis.

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FIGURE LEGENDS

Figure 1 – **The protein levels of Homer1 and CaSR were linked in osteoblasts *in vitro* (A)** (i-xii) Confocal microscopy of osteoblasts transfected with a non-directed control siRNA (siCTRL, i-iv) or siRNA to Homer1 (siHomer1, v-viii) or CaSR (siCaSR, ix-xii). Cells stained for Homer1 (green), CaSR (red) or combined isotype controls and nuclear DAPI (blue) stain (isotype/DAPI). 63x objective, Scale bar=10 μm. **(B)** Western of total osteoblast lysates from cells transfected with siRNA to Homer1 (siHomer1), CaSR (siCaSR) or control sequence (siCTRL). **(C-D)** Densitometry of triplicate bands shown in "B" for (C) CaSR, or (D) Homer1 protein levels. β-actin loading control. ** $p<0.01$, *** $p<0.001$.

Figure 2– **The protein levels of Homer-1b/c and CaSR were linked in bone cells *in vivo*, and the sub-cellular distribution of Homer-1b/c was modulated by CaSR**. **(A-B)** Confocal microscopy of (A) osteocytes embedded in the mineralized matrix or (B) bone lining cells of long bones of wt (CaSR+/+), or CaSR knockout (CaSR-/-) mice, stained for Homer-1b/c (red) or CaSR (green). Cell nuclei (blue) stained by DAPI. 100x objective (scale=5 μm) **(C)** Western blot of protein extracted from mouse long bones from wt (CaSR+/+), heterozygous (CaSR+/-) or CaSR knockout (CaSR-/-). β-actin loading control. **(D)** Densitometry of triplicate blots shown in "C" for Homer-1b/c shown as fold of the level in wt (CaSR+/+). ** $p<0.01$. **(E)** Confocal microscopy of several individual osteocytes from the long bones of CaSR+/+ and CaSR-/- mice stained for Homer-1b/c (red) or CaSR (green). Cell nuclei (blue) stained by DAPI. 100x objective (scale=5 μm). White arrows indicate examples of changes in Homer-1b/c cellular localization between CaSR phenotypes.

Figure 3- Human osteosarcoma cells overexpress Homer1 as well as CaSR and Rictor compared to primary human osteoblasts. Primary human osteoblasts (hOB), or osteosarcoma cells MG63 or SAOS-2 were cultured in 10% DMEM for 48 h and lysed. Equal amounts of total cell protein were then analysed by western blot for CaSR, Homer1, Raptor, Rictor, mTOR or α-tubulin (loading control).

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FIGURE 1

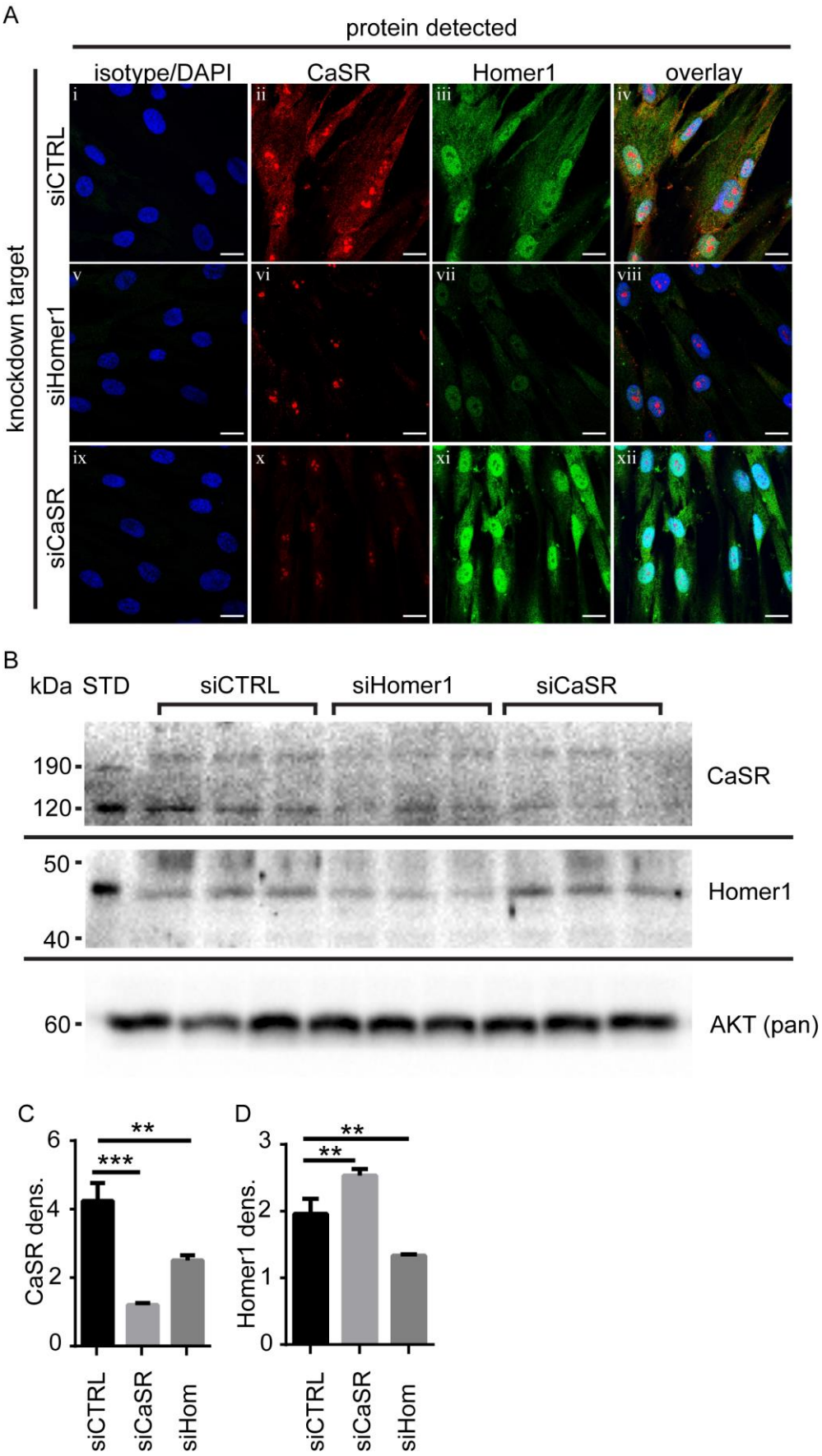

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FIGURE 2

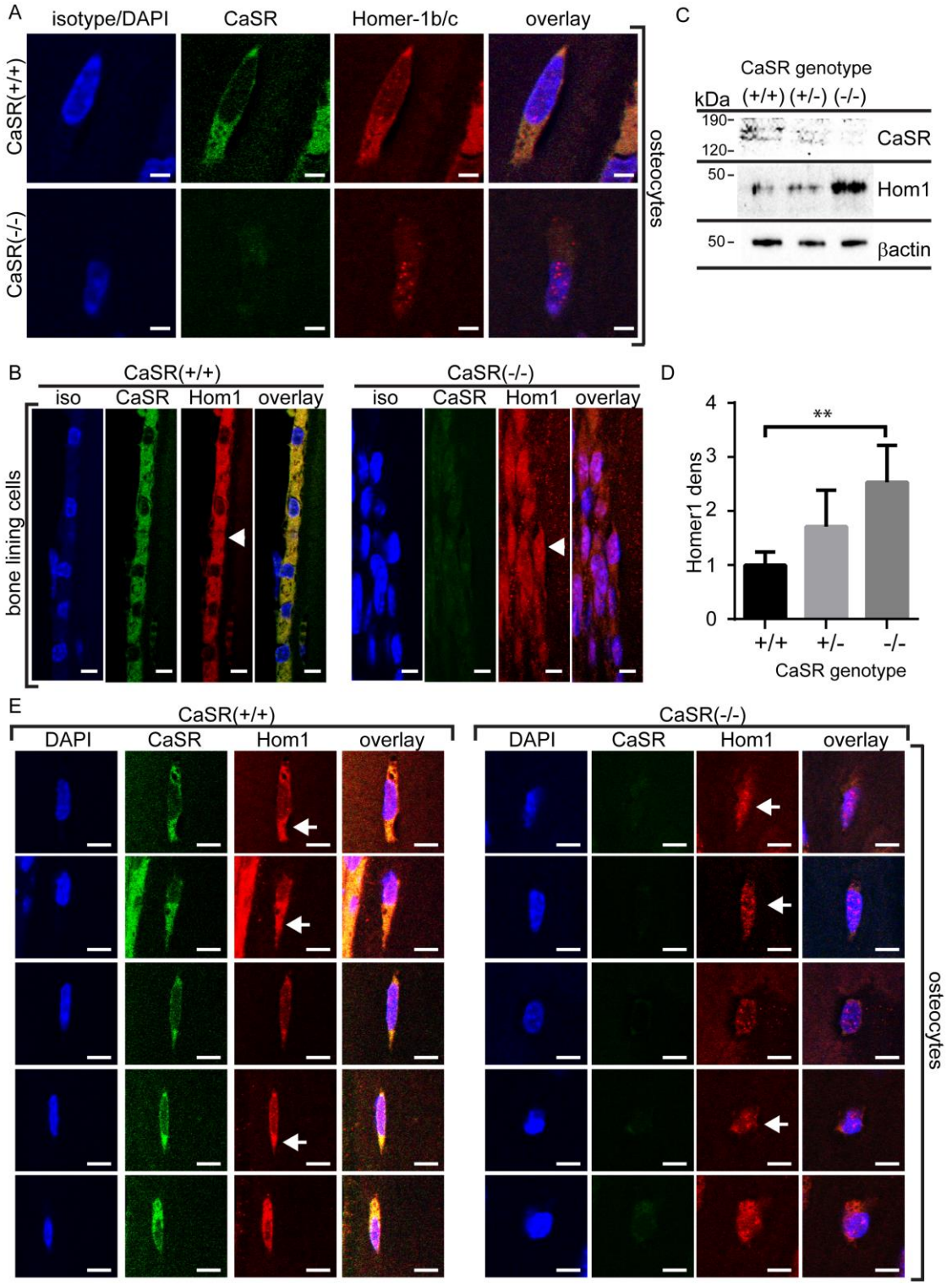

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FIGURE 3