Ionomycin Ameliorates Hypophosphatasia via Rescuing Alkaline Phosphatase Deficiency-mediated L-type Ca\(^{2+}\) Channel Internalization in Mesenchymal Stem Cells

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**One Sentence Summary:** ALP regulates internalization of L-Type Ca\(^{2+}\) Channel of BMSCs in Hypophosphatasia.
Abstract

Loss-of-function mutations in ALPL result in hypophosphatasia (HPP), an inborn error of metabolism that causes skeletal mineralization defect. In adults, main clinical involvement includes early loss of primary or secondary teeth, osteoporosis, bone pain, chondrocalcinosis, and fractures. However, guidelines for the treatment of adults with HPP are not available. Here, we show that ALPL deficiency caused reduction of intracellular Ca$^{2+}$ influx resulting in osteoporotic phenotype due to downregulated osteogenic differentiation and upregulated adipogenic differentiation in both human and mouse BMSCs. To elevate intracellular level of calcium in bone marrow mesenchymal stem cells (BMSCs) by ionomycin treatment rescues the osteoporotic phenotype in alpl$^{+/−}$ mice and BMSC-specific (Prrx1-alpl$^{−/−}$) conditional alpl knockout mice. Mechanistically, ALPL is required to maintain intracellular Ca$^{2+}$ influx by regulating L-type Ca$^{2+}$ channel trafficking via binding to the α2δ subunits, which regulates the internalization of L-type Ca$^{2+}$ channel. Decreased Ca$^{2+}$ flux inactivates Akt/GSK3β/β-catenin signaling pathway that regulates lineage differentiation of BMSCs. This study identifies a previous unknown role of ectoenzyme ALPL in maintenance of calcium channel trafficking to keep stem cell lineage differentiation and bone homeostasis. Accelerating Ca$^{2+}$ flux through L-type Ca$^{2+}$ channel by ionomycin treatment may be a promising therapeutic approach for adult HPP patients.
Introduction

A loss of function mutation in the liver/bone/kidney alkaline phosphatase (ALPL) gene results in life-threatening diseases of hypophosphatasia (HPP) during early developmental periods, featuring hypomineralization of skeleton and teeth (Millan & Whyte, 2016; Whyte, 2016). Adult HPP patients showed early loss of primary or secondary teeth, osteoporosis, bone pain, chondrocalcinosis, and fractures. Our previous study found age-related bone mass loss and marrow fat gain in heterozygous Alpl+/− mice (Liu et al, 2018). Bone marrow mesenchymal stem cells (BMSCs) are multipotent cells capable of differentiating into various cell lineages including osteoblasts and adipocytes. With age, BMSCs are more inclined to undergo differentiation into adipocytes rather than osteoblast, resulting in an increased number of adipocytes and a decreased number of osteoblasts, causing osteoporosis (Li et al, 2018). Our previous study also showed that ALPL governed the osteo-adipogenic balance of BMSCs and prevent cell senescence (Liu et al, 2018). Alkaline phosphatase is known as a ubiquitous plasma membrane-bound enzyme (ectoenzyme), which hydrolyzes several different molecules at physiological (neutral) and alkaline pH, including inorganic pyrophosphate (Russell, 1965), pyridoxal-5-phosphate (the active form of vitamin B6) (Whyte et al, 1985) and nucleotides (Ciancaglini et al, 2010; Scheibe et al, 2000; Zimmermann, 2006). However, the detail mechanism of ALPL causing this age-related osteoporosis is largely unknown.

In severely affected infantile HPP, hypercalcemia and hypercalciuria were often reported as symptoms (Barcia et al, 1997; Belachew et al, 2013). However, it is still not clear why calcium metabolism abnormalities are induced by ALPL mutation, as it plays an important role in generating the inorganic phosphate. Meanwhile, whether the aberrant calcium metabolism involved in age-related osteoporosis of heterozygous Alpl+/− mice is also unclear. It is well accepted that calcium metabolism abnormalities are closely related with calcium channel on the cell surface. Calcium influx is controlled by voltage-gated Ca2+ channels (VGCCs), or agonist-dependent and voltage-independent Ca2+ entry pathways, which are called ‘store-operated’ Ca2+ channels (SOCs). Changes in intracellular Ca2+ concentration ([Ca2+]i) play an essential role in regulating motility, apoptosis, differentiation and many other cellular processes (Berridge et al, 2000). Aberrant intracellular [Ca2+]i leads to loss of Ca2+ homeostasis, which causes abnormal calcium metabolism and bone disorder (Cui et al, 2012; Hoenderop et al, 2003). Several types of Ca2+ channels are reported to regulate intracellular Ca2+ homeostasis in BMSCs and osteoblasts to affect bone repair (Barradas et al, 2012; Jung et al, 2015; Wen et al, 2012). Thus, the regulation of Ca2+ channels at the membrane
plays a central role in BMSC function and bone related diseases. However, whether ALPL modulates Ca\(^{2+}\) channels to maintain Ca\(^{2+}\) homeostasis in BMSCs is unknown.

Currently, specific medical treatment options for HPP are limited to bone-targeted enzyme replacement therapy (asfotase, Strensiq, Alexion), approved for pediatric-onset HPP (Whyte et al, 2012; Whyte et al, 2016). At this time, there are no guidelines for selecting adult patients for treatment, for evaluating the results of treatment, or determining the optimal duration of treatment. In this study, we use human and mouse model to demonstrate that ALPL is required to maintain intracellular Ca\(^{2+}\) influx by regulating L-type Ca\(^{2+}\) channel trafficking via binding to the \(\alpha2\delta\) subunits, which regulates the internalization of L-type Ca\(^{2+}\) channel. This decreased Ca\(^{2+}\) flux downregulates Akt/GSK3β-mediated Wnt/β-catenin signaling in BMSCs, leading to age-related osteoporotic phenotype. Moreover, we found that raising the intracellular level of calcium in BMSCs by ionomycin treatment rescues the osteoporotic phenotype in \(alpl^{+/-}\) and BMSC-specific (\(Prrx1\)-\(alpl^{-/-}\)) conditional \(alpl\) knockout mice, as well as stem cell function of BMSCs from HPP patients, suggesting a new strategy for HPP therapy.

**Results**

**ALPL deficiency caused decreased membrane expression of L-type Ca\(^{2+}\) channel in BMSCs**

As severe ALPL deficiency patients develop hypercalcemia (Barcia et al, 1997; Belachew et al, 2013), we examined the plasma calcium level in \(alpl^{+/-}\) mice and found a marked increase in the level of plasma calcium (Fig S1a). To explore whether ALPL deficiency contributes to abnormal calcium metabolism in BMSCs, we isolated BMSCs from \(alpl^{+/-}\) mice (Fig S1b-e) and examined the cytosolic Ca\(^{2+}\). We found that lentivirus-overexpressing ALPL in \(alpl^{+/-}\) BMSCs is able to increase the cytosolic Ca\(^{2+}\) of \(alpl^{+/-}\) BMSCs (Fig S2a). Ca\(^{2+}\) entry across the plasma membrane occurs via two distinct pathways, SOCs and VGCCs. To test which type Ca\(^{2+}\) channels might be affected in the absence of ALPL, WT and \(alpl^{+/-}\) BMSCs were cultured with 10 nM thapsigargin (TG), a noncompetitive inhibitor capable of raising cytosolic Ca\(^{2+}\) concentration via blocking the ability of the cell to pump Ca\(^{2+}\) into the sarcoplasmic and endoplasmic reticula, to activate plasma membrane Ca\(^{2+}\) channels. Evidently, no significant difference of TG-induced intracellular Ca\(^{2+}\) influx was detected in WT, \(alpl^{+/-}\) and shALP BMSCs (Fig S2b, c). These data suggest that ALPL deficiency affects the VGCC function in BMSCs. The contribution of ALPL to the VGCCs in BMSCs was determined after membrane depolarization using 30 mM KCl. Intracellular Ca\(^{2+}\) imaging
analysis showed that KCl-induced Ca\(^{2+}\) influx was significantly decreased in culture-expanded \textit{alpl}\(^{+/\text{-}}\) and shALP BMSCs compared to WT BMSCs (Fig 1a). Moreover, intracellular Ca\(^{2+}\) imaging analysis showed that KCl-induced Ca\(^{2+}\) influx was not changed in culture-expanded WT, \textit{alpl}\(^{+/\text{-}}\) and shALP BMSCs when treated with 10 mM EGTA (Fig 1b), suggesting that ALPL regulates Ca\(^{2+}\) elevation mainly due to Ca\(^{2+}\) influx with a limited contribution from intracellular Ca\(^{2+}\) storage. Overexpression of ALPL in \textit{alpl}\(^{+/\text{-}}\) BMSCs rescued KCl-induced Ca\(^{2+}\) influx (Fig 1c).

The decrease in Ca\(^{2+}\) currents in \textit{alpl}\(^{+/\text{-}}\) BMSCs could arise from loss of channels at the membrane. The VGCCs comprise 10 subsets, Ca\(_{\text{v}}\)1.1, Ca\(_{\text{v}}\)1.2, Ca\(_{\text{v}}\)1.3, Ca\(_{\text{v}}\)1.4 (L-type), Ca\(_{\text{v}}\)2.1 (P/Q-type), Ca\(_{\text{v}}\)2.2 (N-type), Ca\(_{\text{v}}\)2.3 (R-type), Ca\(_{\text{v}}\)3.1, Ca\(_{\text{v}}\)3.2 and Ca\(_{\text{v}}\)3.3 (T-type) encoded by cacna1s, cacna1c, cacna1d, cacna1f, cacna1a, cacna1b, cacna1e, cacna1g, cacna1h and cacna1l, respectively (Catterall, 2000; Tsien et al, 1988). To identify which subunits of the VGCCs were regulated by ALPL, we measured total protein expression of Ca\(_{\text{v}}\)1.1, Ca\(_{\text{v}}\)1.2, Ca\(_{\text{v}}\)1.3, Ca\(_{\text{v}}\)2.1, Ca\(_{\text{v}}\)2.2, Ca\(_{\text{v}}\)2.3, Ca\(_{\text{v}}\)3.1, Ca\(_{\text{v}}\)3.2 and Ca\(_{\text{v}}\)3.3, as Ca\(_{\text{v}}\)1.4 seems so far to be restricted to the retina. The results showed that total protein expression of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 were decreased significantly in \textit{alpl}\(^{+/\text{-}}\) BMSCs compared to WT BMSCs (Fig 1d). Total protein expression of Ca\(_{\text{v}}\)1.1, Ca\(_{\text{v}}\)2.1, Ca\(_{\text{v}}\)3.1 and Ca\(_{\text{v}}\)3.2 were not changed (Fig 1d and Fig S2d). However, total and membrane expression of Ca\(_{\text{v}}\)2.1, Ca\(_{\text{v}}\)3.1 and Ca\(_{\text{v}}\)3.2 were increased in \textit{alpl}\(^{+/\text{-}}\) BMSCs compared to WT BMSCs (Fig S2d, e). The expression of Ca\(_{\text{v}}\)2.1, Ca\(_{\text{v}}\)3.1 and Ca\(_{\text{v}}\)3.2 in cytoplasm was not changed significantly in \textit{alpl}\(^{+/\text{-}}\) BMSCs compared to WT BMSCs (Fig S2f). Membrane expression of Ca\(_{\text{v}}\)1.1 was increased but the expression in cytoplasm was decreased (Fig 1e, f). Considering the decreased Ca\(^{2+}\) influx in \textit{alpl}\(^{+/\text{-}}\) BMSCs, we focused on Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3. Membrane expression of calcium channel affects the calcium influx, we compared the expression of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 on membrane and in cytoplasm. The results showed that the expression of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 on membrane was decreased in \textit{alpl}\(^{+/\text{-}}\) BMSCs compared to WT BMSCs (Fig 1e). However, the expression of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 in cytoplasm was not changed significantly in \textit{alpl}\(^{+/\text{-}}\) BMSCs compared to WT BMSCs (Fig 1f). Overexpression of ALPL increased the expression of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 on membrane as assayed by western blot (Fig 1g). We also investigated the membrane localization of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 in WT and \textit{alpl}\(^{+/\text{-}}\) BMSCs by confocal laser scanning microscope. The results showed that Ca\(_{\text{v}}\)1.2 (FITC labeled) and Ca\(_{\text{v}}\)1.3 (FITC labeled) were localized on the membrane of WT BMSCs (Fig 1h). However, Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 were absent on the membrane in \textit{alpl}\(^{+/\text{-}}\) BMSCs. Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 were localized on cell membrane after
overexpression of ALPL in alpl+/− BMSCs (Fig 1h). These results suggest that ALPL modulates the member expression of L-type Ca2+ channel, especially CaV1.2 and CaV1.3.

**ALPL maintained MSC osteogenic/adipogenic lineage differentiation via L-type Ca2+ channel**

We knocked down ALPL in BMSCs by siRNA (Fig S3a) and confirmed that ALPL deficiency decreased the osteogenic differentiation and increased the adipogenic differentiation of BMSCs (Fig S3b-e). To address whether the defective osteogenic/adipogenic lineage differentiation of BMSCs with ALPL deficiency owing to the abnormal membrane expression of VGCCs, especially CaV1.2 and CaV1.3, we overexpressed CaV1.2 and CaV1.3 in alpl+/− BMSCs (oeCaV1.2 or oeCaV1.3), and western blot data showed that CaV1.2 and CaV1.3 were increased on membrane of alpl+/− BMSCs after CaV1.2 or CaV1.3 overexpression (oeCaV1.2 or oeCaV1.3) (Fig 2a). KCl-induced Ca2+ influx was elevated after overexpression of CaV1.2 or CaV1.3 in alpl+/− BMSCs (Fig 2b). We also observed the membrane localization of CaV1.2 and CaV1.3 in alpl+/− BMSCs after overexpressing CaV1.2 or CaV1.3 by confocal laser scanning microscope (Fig 2c). We found that overexpression of CaV1.2 or CaV1.3 in alpl+/− BMSCs (oeCaV1.2 or oeCaV1.3) rescued decreased osteogenic differentiation of BMSCs, as evidenced by increased mineralized nodule formation and expression of the osteogenic markers RUNX2 and Sp7 (Fig. 2d, e). In contrast, overexpression of CaV1.2 or CaV1.3 (oeCaV1.2 or oeCaV1.3) decreased adipogenic differentiation of BMSCs, as assessed by Oil red O staining to show decreased numbers of adipocytes and western blot to show downregulation of the adipogenic regulators PPARγ2 and LPL under the adipogenic culture conditions (Fig. 2f, g). However, knockdown of CaV1.2 or CaV1.3 (siCaV1.2 or siCaV1.3) in WT BMSCs caused decreased osteogenic and increased adipogenic differentiation (Fig. 2h-k). These data indicate that ALPL regulates osteogenic and adipogenic lineage differentiation through CaV1.2- and CaV1.3-mediated calcium influx.

**ALPL deficiency promoted the internalization of L-type Ca2+ channel in BMSCs**

To determine whether lack of ALPL leads to internalization, which caused decreased membrane expression of L-type Ca2+ channel in BMSCs, we disrupted endocytosis by expressing a dominant-negative mutant of dynamin 1 (DN-Dyn1), a GTPase required for the formation of endocytic vesicles from the plasma membrane (Praefcke & McMahon, 2004). Expression of DN-Dyn1 in alpl−/− BMSCs prevented the loss of CaV1.2 and CaV1.3 on the cell surface (Fig 3a), providing evidence that lack of ALPL causes internalization of the channels. Western blot analysis
showed that expression levels of membrane Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 in alpl\(^{+/}\) BMSCs were increased after DN-Dyn1 transfection. However, the expression levels of cytoplasm Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 in alpl\(^{-/-}\) BMSCs were not significantly changed after DN-Dyn1 transfection (Fig 3b). Expression of DN-Dyn1 also prevented the decrease of KCl-induced Ca\(^{2+}\) influx in alpl\(^{+/}\) BMSCs (Fig 3c).

We next measured the time course of ALPL-dependent L-type Ca\(^{2+}\) channel internalization. To study this process in live BMSCs, we used Dio to label the cell membrane (FITC labeled) and constructed a plasmid to express Ca\(_{\text{v}}\)1.2 (DsRed-Ca\(_{\text{v}}\)1.2) and transfect the cells. We recorded co-localization region as region of interest (ROI) to record the time-course change of intensity of red fluorescence. DsRed-Ca\(_{\text{v}}\)1.2 signal in alpl\(^{+/}\) BMSCs declined after 10 min, reflecting the decreased membrane expression of L-type Ca\(^{2+}\) channel (Fig 3d, e). However, DsRed-Ca\(_{\text{v}}\)1.2 signal was not changed significantly after 10 min in WT, DN-Dyn1-transfected alpl\(^{+/}\) BMSCs and ALPL-overexpressed alpl\(^{+/}\) BMSCs (Fig 3d, e). We also selected the images of 0 s and 570 s to show the co-localization of DsRed-Ca\(_{\text{v}}\)1.2 with cell membrane of BMSCs. Almost no co-localization region was found in alpl\(^{-/-}\) BMSCs (Fig 3f-i), which suggested ALPL deficiency promoted the internalization of L-type Ca\(^{2+}\) channel. To know whether expression of DN-Dyn1 rescues the differentiation in ALPL deficient BMSCs, we performed osteogenic and adipogenic induction after the transfection. Expression of DN-Dyn1 increased osteogenic differentiation of alpl\(^{+/}\) BMSCs, as evidenced by increased mineralized nodule formation and expression of the osteogenic markers RUNX2 and Sp7 (Fig s4a, b). In contrast, expression of DN-Dyn1 decreased adipogenic differentiation of alpl\(^{+/}\) BMSCs, as assessed by Oil red O staining to show decreased numbers of adipocytes and western blot to show downregulation of the adipogenic regulators PPAR\(\gamma\)2 and LPL under the adipogenic culture conditions (Fig s4c, d).

**ALPL deficiency promoted the internalization of L-type Ca\(^{2+}\) channel via binding to \(\alpha_2\delta\) subunits**

Given that alkaline phosphatase has been reported to hydrolyze inorganic pyrophosphate (PPI) and adenosine triphosphate (ATP), we compared the member expression of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 in BMSCs and BMSCs treated by PPI or ATP. However, exogenous PPI or ATP barely changed the memberane expression of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 in BMSCs (Fig S5a). To further explore the molecular mechanism of ALPL-regulated internalization of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3, we measured the membrane localization of ALPL and calcium channel. We found that Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 were overlapped with ALPL in BMSCs (Fig 4a, upper panel), suggesting the binding of ALPL and L-type Ca\(^{2+}\)
channel. However, no membrane location of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 were found in alpl\textsuperscript{+/-} BMSCs (Fig 4a, lower panel). Several co-localization regions of ALPL and Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 were found in cytoplasm of alpl\textsuperscript{+/-} BMSCs (Fig 4a, lower panel), indicating that ALPL may bind L-type Ca\textsuperscript{2+} channel in cytoplasm. We also used immunoprecipitation to confirm the binding of ALPL and L-type Ca\textsuperscript{2+} channel. Immunoprecipitation using a control antibody did not isolate either protein, but an immunoprecipitation with anti-ALPL resulted in a coimmunoprecipitation with Ca\textsubscript{v}1.2 or Ca\textsubscript{v}1.3 (Fig 4b). In addition, we found anti-Ca\textsubscript{v}1.2 or anti-Ca\textsubscript{v}1.3 enriched ALPL (Fig 4c), suggesting that ALPL directly binds with Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 in BMSCs.

We next investigated what region of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 is important for ALPL-regulated internalization. α\textsubscript{2}δ subunit was indicated in trafficking α1 subunits, which influences internalization of the channels (Canti et al., 2005; Tetreault et al., 2016). Floxed alpl mice with Prrx1::Cre mice were crossed to generate early embryonic MSC-specific (Prrxl-alpl\textsuperscript{-/-}) conditional alpl knockout mice (Fig S5b, c). We isolated the BMSCs from Prrx1-alpl\textsuperscript{-/-} mice (ALPL\textsuperscript{-/-}) and the control alpl\textsuperscript{fl/+} littermates (control), alpl\textsuperscript{+/-} BMSCs showed decreased expression of ALPL and lentivirus-overexpressing ALPL (Leti-alp) treatment elevated the membrane expression of ALPL (Fig S5d). To explore whether ALPL interacted with α\textsubscript{2}δ to regulate the internalization of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3, we expressed ALPL with α\textsubscript{2}δ or mutant α\textsubscript{2}δ in alpl\textsuperscript{+/-} BMSCs and examined the membrane expression of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3. alpl\textsuperscript{+/-} BMSCs were isolated from Prrx1-alpl\textsuperscript{-/-} mice and showed no expression of ALPL, Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 on the membrane (Fig 4d). The membrane expression of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 was increased and ALPL was co-localized with Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 after transfected with ALPL and α\textsubscript{2}δ (Fig 4d). However, the membrane expression of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 was not increased and ALPL was not co-localized with Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 after transfected with ALPL and mutant α\textsubscript{2}δ (Fig 4d). Western blot analysis also confirmed that the membrane expression of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 was increased after transfected with ALPL and α\textsubscript{2}δ (Fig 4e). However, no membrane expression of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 was found in alpl\textsuperscript{+/-} BMSCs and alpl\textsuperscript{+/-} BMSCs transfected with ALPL and mutant α\textsubscript{2}δ (Fig 4e). Immunoprecipitation using a control antibody did not isolate either protein in WT and alpl\textsuperscript{+/-} BMSCs, but an immunoprecipitation with anti-α2δ resulted in a coimmunoprecipitation with ALPL in alpl\textsuperscript{fl/+} BMSCs (Fig 4f). To confirm that ALPL interacts with α\textsubscript{2}δ subunits and thus regulates the lineage differentiation of BMSCs, we transfected alpl\textsuperscript{+/-} BMSCs with α\textsubscript{2}δ or mutant α\textsubscript{2}δ and assessed their osteogenic or adipogenic induction. alpl\textsuperscript{+/-} BMSCs transfected with mutant α\textsubscript{2}δ
showed decreased osteogenic differentiation and increased adipogenic differentiation compared to alpl+/− BMSCs transfected with αδ (Fig S5e-h).

ALPL deficiency caused aberrant lineage differentiation of BMSCs through Wnt/β-catenin Pathway.

In order to examine how ALPL-deficiency-induced reduction of Ca²⁺ influx affects the osteogenic and adipogenic differentiation of BMSCs, we analyzed three Ca²⁺ downstream pathways, including PKC/Erk, PI3K/Akt/GSK3β, and CaMKII/calcineurine A cascades, which are closely linked to Ca²⁺-associated regulation of osteogenic differentiation. We found that the expression level of p-Akt significantly decreased along with the reduction of p-GSK3β in alpl+/− BMSCs and BMSCs transfected with shALP (Fig 5a). However, PKC/Erk and CaMKII/calcineurine A pathways were not changed significantly in alpl+/− BMSCs and BMSCs transfected with shALP compared to WT BMSCs (Fig S6a). Because the decrease of GSK3β phosphorylation inhibits the nuclear translocation of β-catenin, which regulates osteogenic and adipogenic differentiation of BMSCs, we examined the expression levels of total and active β-catenin. We found that the expression of active β-catenin was decreased in both alpl+/− BMSCs and BMSCs transfected with shALP (Fig 5a). Moreover, when we overexpressed ALPL in alpl+/− BMSCs, the expression of p-Akt, p-GSK3β and active β-catenin was all increased to the level similar to WT BMSCs (Fig 5b). When we overexpressed Cav1.2 or Cav1.3 in alpl+/− BMSCs, the expression of p-Akt, p-GSK3β and active β-catenin was all increased (Fig 5c). In addition, expression of DN-Dyn1 in alpl+/− BMSCs also increased the expression of p-Akt, p-GSK3β and active β-catenin compared to alpl+/− BMSCs (Fig 5d). Altogether, these data indicate that ALPL regulates Ca²⁺ influx to affect p-Akt and p-GSK3β expression, and subsequently target on the Wnt/β-catenin pathway in BMSCs.

To further elucidate whether ALPL regulates the osteogenic and adipogenic differentiation through the Akt/GSK3β/Wnt/β-catenin pathway, we used activators for Akt phosphorylation (sc79) and GSK3β phosphorylation (LiCl) to treat alpl+/− BMSCs and found that sc79 and LiCl treatment increased the osteogenic differentiation, as evidenced by increased mineralized nodule formation and expression of RUNX2 and Sp7 (Fig 5e, f). In contrast, sc79 and LiCl treatment decreased adipogenic differentiation of alpl+/− BMSCs, as assessed by Oil red O staining to show decreased number of adipocytes and western blot to show downregulation of PPARγ2 and LPL under adipogenic culture conditions (Fig 5g, h). Moreover, we also overexpressed β-catenin (oeβ-cat) in alpl+/−
BMSCs (Fig S6b) and found rescued lineage differentiation in alpl+/− BMSCs, as evidenced by the increased osteogenic differentiation and decreased adipogenic differentiation (Fig 5i-l).

Raising the intracellular level of calcium by ionomycin rescued ALPL deficiency-induced age-related osteoporosis

Ionomycin was reported to cause a robust rise of Ca\(^{2+}\) influx and may inhibited calcium channel endocytosis (Green et al, 2007). We confirmed that ionomycin treatment increased the calcium influx and the membrane expression of Ca\(_V\)1.2 and Ca\(_V\)1.3 of alpl+/− BMSCs (Fig S7a, b). MicroCT and histological analyses showed that bone mineral density (BMD), bone volume/total volume (BV/TV), and distal femoral trabecular bone structure (Tb.N) in 3-month-old alpl+/− mice were markedly decreased compared to the control WT littermates (Fig. 6a, b). We treated alpl+/− mice with ionomycin, which caused a robust rise of Ca\(^{2+}\) influx in cells (Green et al, 2007). MicroCT and histological analyses showed that BMD, BV/TV and Tb.N in 3-month-old alpl+/− mice treated by ionomycin were markedly increased compared to the alpl+/− mice (Fig. 6a, b). To observe alteration of osteogenic/adipogenic lineage differentiation in vivo, we examined the number of adipocytes in bone marrow of WT, alpl+/− mice and alpl+/− mice treated by ionomycin. Interestingly, Oil red O staining showed that the number of adipocytes in alpl+/− mice bone marrow was markedly increased compared to WT littermates (Fig. 6c), indicating that alpl deficiency increased adipogenic lineage differentiation. However, the number of adipocytes in alpl+/− mice bone marrow after ionomycin treatment was markedly decreased compared to alpl+/− mice (Fig. 6c). To confirm that alpl deficiency directly contributes to the decreased osteogenesis, a calcein double labeling analysis was used to show decreased bone formation rate in alpl+/− mice but elevated bone formation rate in alpl+/− mice treated by ionomycin (Fig. 6d).

Moreover, we found that intracellular level of Ca\(^{2+}\) in alpl+/− BMSCs was decreased compared to WT BMSCs and ionomycin treatment elevated the intracellular level of Ca\(^{2+}\) in alpl+/− BMSCs (Fig. 6e). We found impaired osteogenic differentiation and increased adipogenic differentiation of alpl+/− BMSCs, as evidenced by decreased mineralized nodule formation and elevated numbers of adipocytes (Fig. 6f, h). The deceased expression of the osteogenic markers RUNX2 and Sp7 and increased expression of the adipogenic regulators PPARγ2 and LPL were assayed by western bolt (Fig. 6g, i). Ionomycin treatment increased the osteogenic differentiation and decreased adipogenic differentiation of alpl+/− BMSCs (Fig. 6f-i).
Ionomycin treatment rescued osteoporosis in BMSC-specific conditional alpl knockout mice

To further confirm whether ALPL deficiency in BMSCs caused altered osteogenesis and adipogenesis in vivo, we found BV/TV, and Tb.N in 3-month-old Prx1-alpl<sup>−/−</sup> mice were markedly decreased compared to the control alpl<sup>+/+</sup> littermates (Fig. 7a, b). Floxed alpl littermates (alpl<sup>fl/fl</sup>) were used as the controls. MicroCT and histological analyses showed that BMD, Moreover, BMD, BV/TV, and Tb.N in 3-month-old Prx1-alpl<sup>−/−</sup> mice treated by ionomycin were markedly increased compared to the Prx1-alpl<sup>−/−</sup> mice (Fig. 7a, b). To further observe alteration of osteogenic/adipogenic lineage differentiation in BMSCs, we examined the number of adipocytes in bone marrow of alpl<sup>fl/fl</sup>, Prx1-alpl<sup>−/−</sup> mice and Prx1-alpl<sup>+/+</sup> mice treated by ionomycin and found that Oil red O staining showed that the number of adipocytes in Prx1-alpl<sup>−/−</sup> bone marrow was markedly increased compared to control alpl<sup>fl/fl</sup> littermates (Fig. 7c). However, the number of adipocytes in Prx1-alpl<sup>−/−</sup> bone marrow after ionomycin treatment was markedly decreased compared to Prx1-alpl<sup>−/−</sup> mice (Fig. 7c). Calcein double labeling analysis showed decreased bone formation rate in Prx1-alpl<sup>−/−</sup> mice relative to control alpl<sup>+/+</sup> mice (Fig. 7d). Ionomycin treatment reversed the impaired osteogenesis in Prx1-alpl<sup>−/−</sup> mice. Moreover, the serum levels of RANKL and OPG were not significantly changed, as assessed by ELISA (Fig S7c, d), suggesting that osteoclasts may not be altered in Prx1-alpl<sup>−/−</sup> mice. Intracellular level of Ca<sup>2+</sup> in alpl<sup>−/−</sup> BMSCs was decreased compared to control BMSCs and ionomycin treatment elevated the intracellular level of Ca<sup>2+</sup> in alpl<sup>−/−</sup> BMSCs (Fig. 7e). In addition, BMSCs from Prx1-alpl<sup>−/−</sup> mice showed decreased osteogenic differentiation and increased adipogenic differentiation compared to BMSCs from alpl<sup>fl/fl</sup> mice (Fig. 7f-i). BMSCs from Prx1-alpl<sup>−/−</sup> mice treated by ionomycin showed increased osteogenic and decreased adipogenic differentiation (Fig. 7f-i). These results indicated that ALPL deficiency in BMSCs induces age-related osteoporosis phenotype and ionomycin treatment reversed the osteoporosis phenotype.

ALPL deficiency promoted the internalization of L-type Ca<sup>2+</sup> channel in HPP patient derived BMSCs

We also collected bone marrow BMSCs from two HPP patients with mutation in ALPL gene (A1 and A2, Table S1). The expression of ALPL on membrane and in cytoplasm was decreased in BMSCs from two HPP patients compared to normal human bone marrow BMSCs (control) (Fig 8a). We further confirmed that whether lack of ALPL results in decreased KCl-induced Ca<sup>2+</sup> influx in A1 and A2 BMSCs. KCl-induced Ca<sup>2+</sup> influx was significantly decreased in culture-expanded A1 and A2 BMSCs compared to control BMSCs (Fig 8b) and overexpression of ALPL elevated KCl-induced Ca<sup>2+</sup> influx (Fig 8c, d) in A1 and A2 BMSCs. Moreover, we also
found that expression of DN-Dyn1 prevented the decrease of KCl-induced Ca\(^{2+}\) influx in A1 and A2 BMSCs (Fig 8c, d). Overexpression of ALPL or expression of DN-Dyn1 in A1 and A2 BMSCs increased the membrane expression of Ca\(_{v}\)1.2 and Ca\(_{v}\)1.3 as the confocal images showed (Fig 8e), suggesting that ALPL deficiency causes channel internalization of Ca\(_{v}\)1.2 and Ca\(_{v}\)1.3 in human model. To confirm that ALPL regulates the lineage differentiation of BMSCs, we overexpressed ALPL in A1 and A2 BMSCs and assessed their osteogenic or adipogenic ability after induction. A1 and A2 BMSCs transfected with ALPL vector showed increased osteogenic differentiation and decreased adipogenic differentiation (Fig S8a-d). All of the above data show that ALPL maintains lineage differentiation of MSCs through directly binding to \(\alpha 2\delta\) subunit of L-type Ca\(^{2+}\) channels and inhibiting the internalization of L-type Ca\(^{2+}\) channels, thus increases Ca\(^{2+}\) influx (Fig S9).

**Discussion**

The deficiency of HPP could be ameliorated by bone anabolic and/or enzyme replacement treatment strategies thus far. Bone anabolic treatment such as recombinant human parathyroid hormone (PTH) analogs showed controversial treatment efficacy (Seefried et al, 2017). Asfotase alfa (Strensiq, Alexion), a bone-targeted enzyme replacement therapy, was approved for long-term treatment of pediatric-onset HPP in the United States, Europe, Canada, and Japan. However, there are no guidelines for selecting adult patients for treatment, for evaluating the results of treatment, or determining the optimal duration of treatment at this time. These patients also can develop secondary osteoporosis, bone marrow edema, and delayed fracture healing or difficulties with implant failure (Seefried et al, 2017). Thus, there is an urgent need for identifying further bone targeted treatment options for such adult HPP patients.

Our previous study showed that Alpl deficiency results in premature bone ageing characterized by bone mass loss and parallel marrow fat gain. Although a pivotal role for ALPL in skeletal matrix mineralization is established, the mechanism of differentiation of BMSCs regulated by ALPL remains uncertain. Hypercalcemia was found in severely affected infantile HPP, which suggests that ALPL may modulate calcium homeostasis. In this study, we found that raising the intracellular level of calcium in BMSCs by ionomycin rescued ALPL deficiency caused age-related osteoporosis, which suggest that targeting the calcium channel is a new approach for adult HPP treatment.
Moreover, our study showed a new function of ALPL in controlling the Ca\textsuperscript{2+} influx through regulating the internalization of calcium channels, which balanced the osteogenic and adipogenic differentiation of BMSCs.

Voltage-dependent calcium channel are an important route for the entry of Ca\textsuperscript{2+} into cells upon membrane depolarization to regulate the calcium homeostasis. Previous study showed that VGCCs in BMSCs and osteoblast regulate bone formation and manipulating VGCCs promotes bone repair (Sun et al, 2012; Zhang et al, 2016). Of note, the long-lasting voltage-gated calcium channel (L-VGCC), a major channel of calcium influx, is a part of the high-voltage activated family of voltage-gated calcium channel. L-type calcium channel is considered to play an important role in regulating BMSCs function (Barradas et al, 2012; Wen et al, 2012). L-VGCCs, which consist of four subunits (Ca\textsubscript{V}1.1, Ca\textsubscript{V}1.2, Ca\textsubscript{V}1.3 and Ca\textsubscript{V}1.4), are expressed and distributed in different tissues (Snutch & Reiner, 1992). Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 constitute the major fraction of L-type calcium channels in mammals (Hell et al, 1993; Ludwig et al, 1997; Takimoto et al, 1997). However, the regulation of Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 in BMSCs are still unknown. Here we found that lack of ALPL caused decreased number of Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 in the membrane and decreased Ca\textsuperscript{2+} influx in BMSCs, which leads to the aberrant lineage differentiation of BMSCs.

The involvement of ALPL in channel internalization leads to a change in the number of Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 at the cell surface. Although several other proteins have been found to regulate the membrane expression of VGCCs, including calmodulin, Akap15, Akap9 and eIF3e, and these associations play an important role in connecting VGCCs and intracellular signaling pathways (Green et al, 2007). Of these proteins, ALPL is unusual in that it is an ectoenzyme, which hydrolyzes several substrates. Our study showed a new function of ALPL in controlling the Ca\textsuperscript{2+} influx through regulating the internalization of Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 in BMSCs. The results suggest that ectoenzymes on the cell membrane may bind channels in the cell and regulate the trafficking of channels. Precisely how ALPL regulates L-type Ca\textsuperscript{2+} channel trafficking is unclear. Our co-localization data indicates that there is a direct contact between ALPL and L-type Ca\textsuperscript{2+} channel. \(\alpha2\delta\) subunit of L-type Ca\textsuperscript{2+} channel is responsible for regulating the trafficking of channels (Canti et al, 2005; Tetreault et al, 2016). We further demonstrate that ALPL may bind to the \(\alpha2\delta\) subunit to regulate L-type Ca\textsuperscript{2+} channel trafficking, as the mutation of \(\alpha2\delta\) subunit prevents the membrane expression of Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3. The conditional binding of ALPL to Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 suggests that the composition of Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 protein complex with ALPL may be an important factor in channel regulation.
Skeletal defects in HPP include rickets, osteomalacia, fractures, bone pain and various dental defects (Foster et al, 2013; Foster et al, 2014). To understand the physiological role of ALPL and evaluate the potential treatments, several lines of ALPL knock-out mouse were generated (Narisawa et al, 1997; Waymire et al, 1995). Homozygous mice show severe bone disease, but they often die before puberty (Foster et al, 2017). However, here we found that ALPL deficiency in BMSCs caused decreased osteogenic differentiation and increased adipogenic differentiation. alpl\(^{+/−}\) mouse model phenocopies the adult HPP, and these mice showed inhibited bone formation but increased adipose tissue in the bone matrix. Moreover, the bone formation was inhibited when we generate alpl conditional knockout mice of BMSCs, which was consistent with the recent reports (Foster et al, 2017). Recent data have shown that increased marrow adipose tissue is correlated with dysfunction of bone and hematopoietic regeneration (Ambrosi et al, 2017). We also found that bone formation was inhibited but the adipose tissue was increased in the bone matrix of alpl conditional knockout mice, which suggests that ALPL regulates the osteogenic/adipogenic differentiation of BMSCs and causes the osteoporosis of HPP.

Our finding of the involvement of ALPL in calcium homeostasis revealed the molecular mechanism underlining the balance of BMSCs osteogenic and adipogenic differentiation. The change of Ca\(^{2+}\) influx in BMSCs by H\(_2\)S regulates the osteogenic differentiation through PCK/Erk/Wnt pathway (Liu et al, 2014). Here we demonstrated that Akt/GSK3β/Wnt/β-catenin pathway was the downstream of ALPL regulating Ca\(^{2+}\) influx. Akt/GSK3β/Wnt/β-catenin pathway further balanced the osteogenic and adipogenic differentiation of BMSCs and the bone formation. In our study, we found that ALPL was required to maintain intracellular Ca\(^{2+}\) influx by regulating internalization of L-type Ca\(^{2+}\) channel via binding to the α2δ subunits in BMSCs. Altered intracellular Ca\(^{2+}\) influx caused by ALPL deficiency results in osteoporotic phenotype due to downregulated osteogenic differentiation and upregulated adipogenic differentiation in both human and mouse BMSCs. Inhibition of calcium channel internalization by ionomycin increased the calcium influx enhanced the bone formation of alpl\(^{+/−}\) mice and Prrx1-alpl\(^{+/−}\) mice, suggesting that targeting calcium channel internalization is a potential treatment strategy for adult HPP patients.

**Materials and Methods**
**MICE.** C57BL/6J and B6.129S7-Alpl<sup>tm1Sor</sup>/J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). We produced mice that were homozygous for ALPL<sup>flo</sup> using CRISPR/Cas9 system, an ALPL allele in which the DNA segment includes exons 3 and 4 was flanked by loxP sites. By coinjection of Cas9/sgRNAs and ALPL targeting vector into zygote, we achieved ALPL-floxed heterozygous mice. To generate a tissue-specific Cre-mediated ALPL knockout model, Prx1-Cre (C57BL/6-Prx1<sup>tm1(iCre)</sup>/Bcgen (Beijing Biocytogen Co., Ltd. Beijing, China) mice were mated with mice heterozygous for the ALPL-floxed allele. The offspring inherited Prx1-Cre and two ALPL-floxed alleles. Routine mouse genotyping was performed by PCR. The following primer pairs were used for Cre and floxed alleles: Prx1<sup>iCre</sup> primer 1: AGCGTTTGGTGGATTGCAGC; Prx1<sup>iCre</sup> primer 2: AGTCCCGGTGACTCCAGCAG; Prx1<sup>iCre</sup> primer 3: TGGTGCACAGTCAGGTTG; ALPL-3′loxP-F: CCTGCTACCTGCTTGCAATGG; ALPL-3′loxP-R: AGGACACCAAAGACCAGGACACTA. All animal experiments were performed under institutionally approved protocols for the use of animal research (University of Fourth Military Medical University protocol number k9-024).

**Isolation of mouse bone marrow mesenchymal stem cells.** Bone marrow cells were flushed from the bone cavities of mouse femurs and tibias with 2% heat-inactivated fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Zhejiang, China) in PBS. A single-cell suspension of all nucleated cells was obtained by passing all bone marrow cells through a 70 μm cell strainer (BD Biosciences, San Diego, CA, USA). All the single cells (1 × 10<sup>6</sup>) were seeded in 100 mm culture dishes (Corning, NY, USA) and initially incubated for 48 h at 37 °C and 5% CO<sub>2</sub>. To eliminate the non-adherent cells, the cultures were washed with PBS twice on the second day. The attached cells were cultured for 16 days with alpha minimum essential medium (α-MEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 20% FBS, 2 mM l-glutamine (Invitrogen Life Technology, Carlsbad, CA, USA), 55 μM 2-mercaptoethanol (Invitrogen), 100 U ml<sup>−1</sup> penicillin, and 100 μg ml<sup>−1</sup> streptomycin (Invitrogen). To confirm mesenchymal stem cell character, we used flow cytometric analysis to show that these BMSCs were positive for Sca-1, CD44 and CD73, and negative for CD34 and CD45 (BD Biosciences, San Diego, CA, USA).

**Calcium imaging.** Calcium imaging was performed using the confocal laser microscopy (Zeiss, Oberkochen FV1000, Germany). Intracellular Ca<sup>2+<sup> level ([Ca<sup>2+</sup>]<sub>i</sub>) was represented by the Fluo-3 fluorescence intensity as described previously (Merritt et al, 1990). Briefly, BMSCs were cultured in 12-well plate and incubated with 5 μM
Fluo-3/AM dye (Invitrogen, Life Technology, Carlsbad, CA, USA) for 30 min in α-MEM at 37 °C. BMSCs were again washed for three times with calibrated EGTA/Ca^{2+} solutions. KCl (30 mM) or TG (20 μM, Sigma-Aldrich, St. Louis., MO, USA) was added to test which type of calcium channel was affected. Images were collected every 4 s at 2 Hz with excitation at 488 nm and emission at 530 nm. Data are presented as Fluo-3 fluorescence intensity increase ratio: \( R = \frac{\Delta F}{F_0} \), where \( \Delta F = F - F_0 \). F is the fluorescence value detected and \( F_0 \) is the minimum fluorescence value.

Confocal Microscopy. Confocal images were acquired with a Zeiss Oberkochen FV1000 confocal laser scanning microscope, using ax×60 oilimmersion objective. BMSCs were fixed with 3.7% paraformaldehyde in distilled water at 4°C for 10 min, incubated overnight with primary antibodies, followed by secondary antibodies for 1 hr. The nucleiuses were stained with 1 μg/ml Hoechst 33342. Images were acquired using an argon laser (excitation, 488 nm; emission, BP505–530 nm emission filter) for FITC-labeled-Cav1.2 or Cav1.3, a UV laser for excitation and a BP385–470 nm emission filter for Hoechst 33342, and a He-Ne laser (excitation, 543 nm; emission filter, LP650nm) for Cy3-labeled-ALPL. \( 1 \times 10^5 \) BMSCs were plated onto coverslips, the cells were treated with 10 μM ATP or 10 μM ppi or 1μg/mL ionomycin for 1 h at the next day, and then for immunofluorescence staining of Cav1.2 or Cav1.3. Plasma membrane localization of Cav1.2 or Cav1.3 in BMSCs stained with anti-Cav1.2 or anti-Cav1.3 antibody was recorded for more than 10 cells. Co-localization of L-type Ca^{2+} channel (Cav1.2 or Cav1.3) and ALPL were also observed using laser scanning confocal microscope with images obtained using FV10-ASW Viewer 4.2 (Zeiss, OberkochenFV1000, Germany).

To record the time-course change of internalization of Cav1.2, plasmid encoding DsRed-Cav1.2 was generated and transfected into the BMSCs. The plasmid was constructed by introducing a DsRed segment into the Cav1.2 plasmid (Addgene plasmid # 26572) according to the instruction of the ClonExpress® II One Step Cloning Kit (Vazyme, Nanjing, China). Dio (Thermo Fisher Scientific, MA, USA) was used to label the cell membrane and DAPI (Thermo Fisher Scientific) was used to label nucleus. Average co-localization intensity was determined by selecting a region of interest (ROI) corresponding to the cell’s footprint in the first image and measuring the average intensity in that region over the entire time course. ROI was visually selected in a region of the Dio labeled plasma membrane. Cells that moved out of this ROI were excluded from analysis. The dwell time of channels at the membrane was measured for more than 15 ROIs from at least five cells per condition. We recorded the time dependent red fluorescence...
intensity of these regions. Quantitative data was presented as fluorescence intensity increase ratio: \( R = \frac{F}{F_0} \). \( F \) is the fluorescence value detected and \( F_0 \) is the minimum fluorescence value.

**Cell Surface Protein Isolation.** The expression level of \( \text{Ca}^{2+} \) channels in the surface membrane was determined by isolating cell surface proteins using Membrane and Cytosol Protein Extraction Kit (Beyotime, Shanghai, China). The cells were lysed using lysis buffer containing 1 mM EDTA, 1 mM dithiothreitol, complete protease inhibitors and 1 mM PMSF. The homogenate was centrifuged for 10 min at 700g to remove the nuclear fraction. The supernatant was then centrifuged for 30 min at 14,000g and 4°C to obtain the cytoplasmic and membrane fractions. The protein concentrations were measured by the Bradford Protein Assay Kit (Beyotime, Shanghai, China). Then equal amounts of the cytoplasmic and membrane proteins were saved as direct input for immunoblot experiments.

**Osteogenic differentiation.** BMSCs were cultured under osteogenic culture conditions containing 2 mM \( \beta \)-glycerophosphate (Sigma-Aldrich, St. Louis., MO, USA), 100 \( \mu \)M L-ascorbic acid 2-phosphate (MP Biomedicals, Irvine, CA, USA), and 10 nM dexamethasone (Sigma-Aldrich, St. Louis., MO, USA) in the growth medium. Two weeks after induction, staining was performed to detect matrix mineralization with 1% Alizarin Red S (Sigma-Aldrich) for 3 min at room temperature. 10 \( \mu \)M SC79 or 10 mM LicL were added to the osteogenic culture medium for induction. The stained areas were quantified using NIH Image J software and shown as a percentage of the total area.

**Adipogenic differentiation.** For adipogenic induction, 500 nM isobutylm ethylxanthine (MP Biomedicals, Irvine, CA, USA), 60 \( \mu \)M indomethacin (Sigma-Aldrich), 500 nM hydrocortisone (MP Biomedicals, Irvine, CA, USA), 10 \( \mu \)g/ml insulin (Sigma-Aldrich), and 100 nM L-ascorbicacid phosphate were added into the growth medium. 10 \( \mu \)M SC79 or 10 mM LicL were added to the adipogenic culture medium for induction. After 7 days, the cultured cells were stained with Oil Red-O (Sigma-Aldrich), and positive cells were quantified under microscopy and shown as a percentage of the total cells.
**Western immunoblotting.** Cells were lysed in M-PER mammalian protein extraction reagent (Thermo, MA, USA) with protease and phosphatase inhibitors (Roche, Basel, Switzerland), and proteins were quantified using protein assays (Bio-Rad Laboratories, Shanghai, China). 20 μg of proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 0.1% Tween 20 and 5% BSA for 1 h before overnight incubation with the primary antibody diluted in blocking solution. Antibody to mouse ALP was purchased from R&D Systems, antibodies to Cav1.1, Cav1.2, Cav1.3, Cav2.1, Cav2.2, Cav3.1, Cav3.2, Cav3.3 and Cavα2δ1 were purchased from alomone labs (Alomone, Jerusalem, Israel). Antibodies to mouse phospho-Erk1/2 (Thr202/Tyr204), Erk1/2, GSK-3β, phospho-GSK-3β, phospho-AKT, β-catenin, RUNX2, SP7, PPARγ, LPL, phospho-PKC, PKC, phospho-CamkII, AKT were purchased from Abcam (Cambridge, UK). Antibodies to mouse CamkII and active β-catenin were obtained from Millipore (Billerica, MA, USA). Antibody to mouse β-actin was purchased from Boster (Wuhan, China). The membranes were incubated for 1 h in HRP-conjugated secondary antibody diluted at 1:40,000 in blocking solution. Immunoreactive proteins were detected using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). The intensity of bands was measured using NIH ImageJ software and normalized to β-actin.

**Co-Immunoprecipitation.** In order to test whether ALPL and L-VGCC (Cav1.2 or Cav1.3) bind each other in cells, co-immunoprecipitation was performed as previously described (J et al, 2008). The cells were completely lysed by Cell lysis buffer for Western and IP (Beyotime, Shanghai, China). The lysates were incubated with primary antibodies for overnight at 4°C and then protein A/G magnetic beads (Millipore, USA) were added for 2 h at 4°C. Immuno-complexes were washed three times with PBS containing 0.1% Tween-20 and subsequently subjected to western blot analysis.

**Plasmids construction.** For shRNA knockdown experiments, we constructed shALPL lentiviral vector according to the protocol of pLKO.1-TRC Cloning Vector (Moffat et al, 2006). In brief, to generate oligos for cloning into pLKO.1, we synthesized the following oligonucleotides: forward oligo: CCGGGCAGTATGAATTGAATC GGAACTCGAGTTCCGATTCAATTCTACTGCTTTTTG, reverse oligo: AATTCAAAAAGCAGTATGAATTGAATCGGAACCTCGAGTTCCGATTCAATTCTACTGC.
Then the forward and reverse oligos were annealed and ligated into the pLKO.1 vector, producing a final plasmid that expresses the shRNA of ALPL. To overexpress ALPL in BMSCs, we constructed lentiviral vector with ALPL cDNA. The ALPL (GenBank Accession No. NM_000478.5) cDNA was amplified by PCR using the primer pairs: Forward: 5’-ACTGGATCCTCAAGGATAAAGCAGGTCT-3’; Reverse: 5’-TATCTCGAGTGGGAAAGTTGGCATCTTGTC-3’. And then the ALPL gene was inserted into the vector pENTR™2B (Invitrogen Life Technology, Carlsbad, CA, USA), a Gateway LR recombination reaction between the ALPL clone vector and pLenti6.3/V5-DEST, to generate an expression clone Lenti-ALPL. The cDNA encoding β-catenin (GenBank Accession No. NM_007614.3) was amplified by TaKaRa LA Taq® with GC Buffer (TaKaRa, Japan). Primers were as following: forward: 5’-CGGGGAGGCGAGACGGAGCAC-3’, reverse: 5’-CCAGCCCACCCTCGAGCCCTCTC-3’. And then the restriction enzymes SalI and BamHI were used to clone the above fragment into the backbone vector pIRES2-EGFP (Clonetech, USA).

pDsRed-Cav1.2 was constructed according to the manufacturer’s protocol of the ClonExpress Ultra One Step Cloning Kit (Vazyme, Nanjing, China). In brief, Cav1.2 was amplified by prierms: forward: 5’-GGGGTACCATGGTCAATGAAAACACGAGGAGTCT-3’, reverse: 5’-ATAAGAATGCGGCCGCCTACAGGTTGCTGACGTAGGAC-3’ from pCav1.2 (addgene, Plasmid #26572), then the fragment was cloned into the vector pCav1.2 by restriction enzymes KpnI and NotI to construct a recombinant plasmid pCav1.2-KN. DsRed was amplified by primers: 5’-GGGAGACCCAAGCTGGCTAGCCTGGACAAACACGAGGAGTCTAGC-3’, reverse: 5’-GTTTTCTATGACCATGCTACTGAGCGAGCTGGCATGCG from pLVX-EF1α-DsRed-monomer-N1 (Biovector NTCC Inc., Beijing, China). Finally, the DsRed fragment was ligased with linearized pCav1.2-KN by ExnaseII to construct the pDsRed-Cav1.2.

**Transfection.** To transfect with small interfering RNA, BMSCs (0.5 × 10^6) were seeded on a 6-well culture plate and transfected with Cav1.2 siRNA or Cav1.3 siRNA (Santa Cruz, Dallas, Texas, USA) using X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer’s instructions.

In order to down-regulate or overexpress ALPL in BMSCs, we firstly produced lentivirus, which respectively carried shALPL or ALPL cDNA. The Lentiviral vector and the ViraPower Packaging Mix were cotransfected into the 293T cell line to produce a lentiviral stock, following the protocol provided by the manufacturer. Virus-
containing supernatants were harvested 48 h after transfection, pooled and filtered through 0.45-µm filters. Cells were treated with lentivirus at a multiplicity of infection of 100 at 37°C and 5% CO₂. Plates were swirled every 15 min and fresh medium was added after 1 hour of incubation. The cells were exposed to lentivirus for 48 hours, followed by protein extraction for Western immunoblotting or differentiation induction.

For plasmid transfection of pCatenin-EGFP, pCav1.2 (addgene, Plasmid #26572), pCav1.3 (addgene, Plasmid #49332), DN-Dyn1 (addgene, Plasmid #55795), pCa₉α2δ1(addgene, Plasmid #26575) and pCa₉α2δ1mut (addgene, Plasmid #58730), we operated according to the protocol of X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland). After transfection, cells were used for protein extraction for western immunoblotting or for differentiation induction or for confocal images.

**MicroCT analysis.** Femurs were harvested and analyzed using a desktop micro-CT system (eXplore Locus SP, GE Healthcare, USA). The scanner was set at a voltage of 80 kVp, a current of 80 µA and a resolution of 8 µm per pixel. Cross-sectional images of the femur mid-diaphysis were used to perform three-dimensional histomorphometric analysis of trabecular bone. Cross sectional volumetric bone mineral density (BMD) was measured for the right femur mid-diaphysis with a density phantom. Using 3-dimensional images, a region of interest in the secondary spongiosa was manually drawn near the endocortical surface. Bone volume relative to tissue volume (BV/TV) and trabecular bone number (Tb.N) were assessed as cancellous bone morphometric parameters.

**Histology.** To assess the areas of trabecular bone and bone marrow, femurs and tibias were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis., MO, USA) and then decalcified with 5 % EDTA (pH 7.4), followed by paraffin embedding. The 6 µm paraffin sections were stained with hematoxylin and eosin (H&E) and analyzed using NIH ImageJ software. To label the mineralization fronts, the mice were given intraperitoneal injections of calcein (Sigma-Aldrich, USA, 20 mg/Kg body weight) at day 10 and day 3 before sacrifice. Bone dynamic histomorphometric analyses for MAR were performed according to the standardized nomenclature for bone histomorphometry under fluorescence microscopy (Leica DM 6000B, German).

**In vivo Oil Red O staining.** To assess the adipose tissue in trabecular areas, femurs were fixed in 4% paraformaldehyde and decalcified with 5% EDTA (pH 7.4), followed by cryosectioning. Sections were stained with
Oil Red O, and positive areas were quantified under microscopy and shown as a percentage of the total area. Briefly, sections were washed with 60% isopropanol and incubated in freshly made Oil Red O staining solution for 15 min at room temperature and counterstained with hematoxylin. All reagents for Oil Red O staining were purchased from Sigma-Aldrich.

**Ionomycin treatment.** Ionomycin (Alomone, Jerusalem, Israel) were dissolved in DMSO. For *in vivo* treatment, ionomycin was intraperitoneally administered to 12 weeks *alpl<sup>+/−</sup>* mice and *alpl<sup>−/−</sup>* CKO mice at a dose of 1 mg/kg/day for 28 days. The control mice were treated with vehicle only. After ionomycin treatment, all groups of mice were healthy.

**Statistics.** All experimental group sizes were chosen to ensure adequate statistical power despite the highly variable nature of the studies performed. No animals were excluded, and animals were randomly assigned groups for the studies. Experiments were not performed in a blinded fashion. Data were assessed for normal distribution and similar variance between groups. Comparisons between two groups were performed using independent unpaired two-tailed Student’s *t*-test, and the comparisons between more than two groups were analyzed using one-way ANOVA with the Bonferroni adjustment. *P* values less than 0.05 were considered statistically significant.
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**Author contributions**

B.L., X.H. and Z.D. performed the animal experiments, immunofluorescence staining and immunoblot, cultured cells, collected data, analyzed the data and produced all figures and tables. K.X., collected the cells from patients, W.S. and L.G. helped with the immunofluorescence staining and participated in the animal surgery. S.L., W.L. C.H. and Y.Z. helped with data analysis. S.S. and Y.J. designed the experiments, oversaw the collection of results and data interpretation and drafted the reports. All authors have seen and approved the final version.

**Conflict of interest**

The authors declare that they have no conflict of interest.
Figure Legend

Figure 1. ALPL deficiency caused decreased membrane expression of L-type Ca\(^{2+}\) channel in BMSCs. (a) Ca\(^{2+}\) imaging showed decreased Ca\(^{2+}\) influx of cultured alpl\(^{+/}\) BMSCs and WT BMSCs transfected with shALP (shALP/WT) stimulated with 30 mM KCl for 3 min (n=10). (b) No KCl-induced Ca\(^{2+}\) influx was detected in cultured WT, alpl\(^{+/}\) and shALP/WT BMSCs treated by 10 mM EGTA for 3 min (n=10). (c) Lentivirus-overexpressing ALPL treated alpl\(^{+/}\) (Leti-alpl/alpl\(^{+/}\)) BMSCs showed an elevated Ca\(^{2+}\) influx stimulated with 30 mM KCl for 3 min (n=10). (d, e, f) The expression of Ca\(_{\text{v}}\)1.1, Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 was assessed. alpl\(^{+/}\) BMSCs showed downregulation of total cell expression (d) and membrane expression of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 (e), and no change of cytoplasm expression of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 (f). (g) Leti-alpl/alpl\(^{+/}\) BMSCs showed an elevated membrane expression of ALP, Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3. (h) Representative images of confocal laser scanning microscope showed membrane location of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 in WT and Leti-alpl/alpl\(^{+/}\) BMSCs. Scale bar, 10 μm. Representative results were from three independent experiments. Error bars represent the s.d. from the mean values. *P < 0.05. **P < 0.01. *** P < 0.001.

Figure 2. ALPL maintained MSC osteogenic/adipogenic lineage differentiation via L-type Ca\(^{2+}\) channel. (a) Plasmid-overexpressed Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 of alpl\(^{+/}\) BMSCs showed upregulated membrane expression of both channels by western blot analysis. (b) Ca\(^{2+}\) imaging showed elevated Ca\(^{2+}\) influx of cultured alpl\(^{+/}\) BMSCs transfected with oeCa\(_{\text{v}}\)1.2 or oeCa\(_{\text{v}}\)1.3 stimulated with 30 mM KCl for 3 min (n=10). (c) Representative images of confocal laser scanning microscope showed membrane location of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 in alpl\(^{+/}\) BMSCs transfected with oeCa\(_{\text{v}}\)1.2 or oeCa\(_{\text{v}}\)1.3. Scale bar, 10 μm. (d) Alizarin red staining showed that alpl\(^{+/}\) BMSCs transfected with siCa\(_{\text{v}}\)1.2 or siCa\(_{\text{v}}\)1.3 had a decreased capacity to form mineralized nodules when cultured under osteo-inductive conditions. (e) Western blot analysis showed that BMSCs transfected with siCa\(_{\text{v}}\)1.2 or siCa\(_{\text{v}}\)1.3 expressed decreased levels of the osteogenic related proteins RUNX2 and Sp7. β-actin was used as a protein loading control. (f, g) BMSCs transfected with siCa\(_{\text{v}}\)1.2 or siCa\(_{\text{v}}\)1.3 showed an increased number of Oil red O-positive adipocytes when cultured under adipo-inductive conditions (f) and upregulation of the adipogenic related proteins PPARγ2 and LPL, as assessed by western blot (g). (h, i) Alizarin red staining showed that alpl\(^{+/}\) BMSCs transfected with oeCa\(_{\text{v}}\)1.2 or oeCa\(_{\text{v}}\)1.3 had an increased capacity to form mineralized nodules when cultured under osteo-inductive conditions (h) and upregulation of the osteogenic related proteins RUNX2 and Sp7 (i). (j, k) oeCa\(_{\text{v}}\)1.2 or oeCa\(_{\text{v}}\)1.3 treated alpl\(^{+/}\)
BMSCs showed a decreased number of Oil red O-positive adipocytes when cultured under adipo-inductive conditions (j) and downregulation of the adipogenic related proteins PPARγ2 and LPL, as assessed by western blot (k). Representative results were from three independent experiments. Error bars represent the s.d. from the mean values. *P < 0.05. ** P < 0.01. *** P < 0.001.

**Figure 3.** ALPL deficiency promoted the internalization of L-type Ca²⁺ channel in BMSCs. (a) Representative images of confocal laser scanning microscope showed membrane location of Caᵥ1.2 and Caᵥ1.3 in alpl⁺/⁻ BMSCs transfected with DN-Dyn1. Scale bar, 10 μm. (b) alpl⁺/⁻ BMSCs transfected with DN-Dyn1 showed upregulation of membrane expression of Caᵥ1.2 and Caᵥ1.3 and almost no change of cytoplasm expression of Caᵥ1.2 and Caᵥ1.3, as assessed by western blot. β-actin was used as a protein loading control. (c) Ca²⁺ imaging showed elevated Ca²⁺ influx of cultured alpl⁺/⁻ BMSCs transfected with DN-Dyn1 when stimulated with 30 mM KCl for 3 min (n=10). (d, e) 10 min time-lapse confocal laser scanning microscopy images of WT, alpl⁺/⁻, DN-Dyn1/alpl⁺/⁻ and Leti-alp/alpl⁺/⁻ BMSCs containing DsRed-Caᵥ1.2. Scale bar, 10 μm (d) alpl⁺/⁻ BMSCs showed a pronounced decrease of DsRed-Caᵥ1.2 on the membrane (Dio labeled ROI, n=10) (e). (f-i) Representative images showed co-localization of DsRed-Caᵥ1.2 with Dio labeled cell membrane of BMSCs. WT, DN-Dyn1/alpl⁺/⁻ and Leti-alp/alpl⁺/⁻ BMSCs had co-localization region at 0s and 570s (f, h, i). However, alpl⁺/⁻ BMSCs showed no co-localization at 0s and 570s (g). Representative results were from three independent experiments. Error bars represent the s.d. from the mean values. *P<0.05, ** P < 0.01.

**Figure 4.** ALPL deficiency promoted the internalization of L-type Ca²⁺ channel via binding to α₂δ subunits. (a) Representative images of confocal laser scanning microscope showed membrane co-localization region of ALPL (DsRed) and Caᵥ1.2 (FITC-labeled) or Caᵥ1.3 (FITC-labeled) in WT BMSCs. No membrane co-localization region was found in alpl⁺/⁻ BMSCs. Scale bar, 10 μm. (b) ALPL immunoprecipitated Caᵥ1.2 and Caᵥ1.3. Left lane showed the expression of Caᵥ1.2 and Caᵥ1.3, and right lane showed the levels of Caᵥ1.2 and Caᵥ1.3 following immunoprecipitation with an anti-ALPL antibody. (c) Caᵥ1.2 and Caᵥ1.3 immunoprecipitated ALPL. Left panel showed the expression of ALPL and right panel showed the level of ALPL following immunoprecipitation with anti-Caᵥ1.2 or anti-Caᵥ1.3 antibody. (d) Representative images of confocal laser scanning microscope showed membrane co-localization region of ALPL (Cy3-labeled) and Caᵥ1.2 (FITC-labeled) or Caᵥ1.3 (FITC-labeled) in
alpl−/− BMSCs overexpressed with ALPL and α2δ subunit. No membrane co-localization region was found in alpl−/− BMSCs and alpl−/− BMSCs overexpressed with ALPL and α2δ subunit. Scale bar, 10 μm. (e) Western blot analysis showed membrane expression of CaV1.2 or CaV1.3 in alpl−/− BMSCs overexpressed with ALPL and α2δ subunit. No membrane expression of CaV1.2 or CaV1.3 was found in alpl−/− BMSCs and alpl−/− BMSCs overexpressed with ALPL and mutant α2δ subunit. No significant change of CaV1.2 or CaV1.3 in cytoplasm was found in alpl−/− BMSCs. alpl−/− BMSCs overexpressed with ALPL and mutant α2δ subunit, alpl−/− BMSCs overexpressed with ALPL and α2δ subunit. (f) α2δ immunoprecipitated ALPL. Left panel showed the expression of ALPL and right panel showed the level of ALPL following immunoprecipitation with anti-α2δ antibody in alpβ/ and alpl−/− BMSCs. β-actin was used as a protein loading control. Representative results were from three independent experiments.

Figure 5. ALPL deficiency caused aberrant lineage differentiation of BMSCs through Wnt/β-catenin Pathway. (a) alpl+/− and shALP BMSCs showed significant downregulation of p-AKT, p-GSK3β and active β-catenin compared to WT BMSCs. β-actin was used as a protein loading control. (b) Leti-alp/alpl+/− BMSCs showed significant upregulation of p-AKT, p-GSK3β and active β-catenin compared to alpl+/− BMSCs. (c) alpl+/− BMSCs transfected with oeCaV1.2 or oeCaV1.3 showed significant upregulation of p-AKT, p-GSK3β and active β-catenin. (d) alpl+/− BMSCs transfected with DN-Dyn1 showed significant upregulation of p-AKT, p-GSK3β and active β-catenin. (e, f) Alizarin red staining showed that alpl+/− BMSCs treated by 10 μM sc79 or 10 mM LiCl had an increased capacity to form mineralized nodules when cultured under osteo-inductive conditions (e) and upregulation of the osteogenic related proteins RUNX2 and Sp7 (f). (g, h) alpl+/− BMSCs treated by sc79 or LiCl showed a decreased number of Oil red O-positive adipocytes when cultured under adipo-inductive conditions (g) and downregulation of the adipogenic related proteins PPARγ2 and LPL, as assessed by western blot (h). (i, j) Alizarin red staining showed that alpl+/− BMSCs transfected with plasmid-overexpressed β-catenin (oeβ-cat/ALPL+/−) had an increased capacity to form mineralized nodules when cultured under osteo-inductive conditions (i) and upregulation of the osteogenic related proteins RUNX2 and Sp7 (j). (k, l) alpl+/− BMSCs transfected with plasmid-overexpressed β-catenin (oeβ-cat/ALPL+/−) showed a decreased number of Oil red O-positive adipocytes when cultured under adipo-inductive conditions (k) and downregulation of the adipogenic related proteins PPARγ2 and LPL, as assessed by western blot (l). Representative results were from three independent experiments. Error bars represent the s.d. from the mean values. *P<0.05, ** P < 0.01.
Figure 6. Raising the intracellular level of calcium by ionomycin rescued ALPL deficiency-induced age-related osteoporosis. (a) MicroCT analysis showed that alpl<sup>+/−</sup> mice (n=12) had significantly decreased bone mineral density (BMD), bone volume over total volume (BV/TV), and trabecular number (Tb. N) in trabecular bone (TB) of the distal femur compared to WT littermates and alpl<sup>+/+</sup> mice treated by ionomycin (n=10). (b) H&E staining showed a decreased TB volume (yellow circled area) in the distal femurs of alpl<sup>+/−</sup> mice compared to the WT group and alpl<sup>+/−</sup> mice treated by ionomycin. Scale bar, 1 mm. (c) Representative histological images of distal femurs showed a significantly increased number of adipocytes in alpl<sup>+/−</sup> mouse bone marrow, as assessed by Oil red O staining. Scale bar, 25 μm. (d) Calcein double labeling assay showed a significantly decreased bone formation rate in alpl<sup>+/−</sup> mice compared to the WT and alpl<sup>+/−</sup> mice treated by ionomycin. Scale bar, 25 μm. (e) Intracellular level of Ca<sup>2+</sup> in alpl<sup>+/−</sup> BMSCs was decreased compared to WT BMSCs and ionomycin treatment elevated the intracellular level of Ca<sup>2+</sup> in alpl<sup>+/−</sup> BMSCs. (f) Alizarin red staining showed that alpl<sup>+/−</sup> BMSCs had a decreased capacity to form mineralized nodules compared to WT BMSCs when cultured under osteo-inductive conditions. Ionomycin treatment of alpl<sup>+/−</sup> BMSCs showed an elevated capacity to form mineralized nodules. (g) Western blot analysis showed that alpl<sup>+/−</sup> BMSCs had decreased expression of osteogenic related proteins RUNX2 and Sp7 compared to WT BMSCs and ionomycin treatment of alpl<sup>+/−</sup> BMSCs. β-actin was used as a protein loading control. (h, i) alpl<sup>+/−</sup> BMSCs showed an increased number of Oil red O-positive cells when cultured under adipo-inductive conditions (h) and upregulation of the adipogenic related proteins PPARγ2 and LPL, as assessed by western blot (i) compared to WT BMSCs and ionomycin treatment of alpl<sup>+/−</sup> BMSCs. Representative results were from three independent experiments. Error bars represent the s.d. from the mean values. *P<0.05, ** P < 0.01, *** P < 0.001.

Figure 7. Ionomycin treatment rescued osteoporosis in BMSC-specific conditional alpl knockout mice. (a) MicroCT analysis showed that Prxl-alpl<sup>+/−</sup> mice (n=12) had significantly decreased bone mineral density (BMD), bone volume over total volume (BV/TV), and trabecular number (Tb. N) in trabecular bone (TB) of the distal femur compared to alpl<sup>+/+</sup> littermates and Prxl-alpl<sup>+/−</sup> mice treated by ionomycin (n=10). (b) H&E staining showed a decreased TB volume (yellow circled area) in the distal femurs of Prxl-alpl<sup>+/−</sup> mice compared to the alpl<sup>+/+</sup> control group and Prxl-alpl<sup>+/−</sup> mice treated by ionomycin. Scale bar, 1 mm. (c) Representative histological images of distal femurs showed a significantly increased number of adipocytes in Prxl-alpl<sup>+/−</sup> mouse bone marrow, as assessed by
Oil red O staining. Scale bar, 25 μm. (d) Calcein double labeling assay showed a significantly decreased bone formation rate in Prx1-alpl<sup>−/−</sup> mice compared to the alpl<sup>+/+</sup> controls and Prx1-alpl<sup>−/−</sup> mice treated by ionomycin. Scale bar, 25 μm. (e) Intracellular level of Ca<sup>2+</sup> in alpl<sup>−/−</sup> BMSCs was decreased compared to WT BMSCs and ionomycin treatment elevated the intracellular level of Ca<sup>2+</sup> in alpl<sup>−/−</sup> BMSCs. (f) Alizarin red staining showed that alpl<sup>−/−</sup> BMSCs had a decreased capacity to form mineralized nodules compared to WT BMSCs when cultured under osteo-inductive conditions. BMSCs from Prx1-alpl<sup>−/−</sup> mice treated by ionomycin showed an elevated capacity to form mineralized nodules. (g) Western blot analysis showed that alp<sup>−/−</sup> BMSCs had decreased expression of osteogenic related proteins RUNX2 and Sp7 compared to WT BMSCs and ionomycin treatment of alpl<sup>−/−</sup> BMSCs. β-actin was used as a protein loading control. (h, i) alpl<sup>−/−</sup> BMSCs showed an increased number of Oil red O-positive cells when cultured under adipo-inductive conditions (h) and upregulation of the adipogenic related proteins PPARγ2 and LPL, as assessed by western blot (i) compared to WT BMSCs and ionomycin treatment of alpl<sup>−/−</sup> BMSCs. Representative results were from three independent experiments. Error bars represent the s.d. from the mean values. *P<0.05, ** P < 0.01. *** P < 0.001.

**Figure 8. ALPL deficiency promoted the internalization of L-type Ca<sup>2+</sup> channel in HPP patient derived BMSCs.** (a) The expression of ALPL on membrane and in cytoplasm was decreased in BMSCs from two HPP patients compared to normal human BMSCs. β-actin was used as a protein loading control. (b) Intracellular Ca<sup>2+</sup> imaging analysis showed that KCl-induced Ca<sup>2+</sup> influx was significantly decreased in cultured BMSCs from HPP patients compared to normal human BMSCs. (c) Overexpression of ALPL or transfected with DN-Dyn1 in BMSCs from A1 patient showed elevated KCl-induced Ca<sup>2+</sup> influx. (d) Overexpression of ALPL or transfected with DN-Dyn1 in BMSCs from A2 patient showed elevated KCl-induced Ca<sup>2+</sup> influx. (e) Representative images of confocal laser scanning microscope showed membrane location of Ca<sub>V</sub>1.2 and Ca<sub>V</sub>1.3 in control BMSCs, A1 BMSCs, A2 BMSCs, A1 and A2 BMSCs overexpressed with ALPL, A1 and A2 BMSCs transfected with DN-Dyn1. Scale bar, 10 μm. Representative results were from three independent experiments. Error bars represent the s.d. from the mean values. *P < 0.05. **P < 0.01.
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