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Rational identification of a Cdc42 inhibitor presents a new regimen for long-term hematopoietic stem cell mobilization

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Abstract

Mobilization of hematopoietic stem cells (HSCs) from bone marrow (BM) to peripheral blood (PB) by cytokine granulocyte colony-stimulating factor (G-CSF) or the chemical antagonist of CXCR4, AMD3100, is important in the treatment of blood diseases. Due to clinical conditions of each application, there is a need for continued improvement of HSC mobilization regimens. Previous studies have shown that genetic ablation of the Rho GTPase Cdc42 in HSCs results in their mobilization without affecting survival. Here we rationally identified a **C**dc42 **a**ctivity-**s**pecific **in**hibitor (CASIN) that can bind to Cdc42 with submicromolar affinity and competitively interfere with guanine nucleotide exchange activity. CASIN inhibits intracellular Cdc42 activity specifically and transiently to induce murine hematopoietic stem/progenitor cell egress from the BM by suppressing actin polymerization, adhesion, and directional migration of stem/progenitor cells, conferring Cdc42 knockout phenotypes. We further show that, although, CASIN administration to mice mobilizes similar number of phenotypic HSCs as AMD3100, it produces HSCs with better long-term reconstitution potential than that by AMD3100. Our work validates a specific small molecule inhibitor for Cdc42, and demonstrates that signaling molecules

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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downstream of cytokines and chemokines, such as Cdc42, constitute a useful target for long-term stem cell mobilization.

Introduction

Hematopoietic stem cells (HSCs) are a rare population of cells residing in a unique bone marrow (BM) micro-environment (also known as niche), that undergo a complex but highly ordered hematopoiesis program throughout the life-span of mammals [1–5]. In addition to supporting blood cell development, HSCs are characterized by their self-renewal ability, a property that explains the mostly conserved HSC numbers found in animals [1, 4, 6]. HSC transplantation has become a standard care for the treatment of many hematologic malignancies, including chemotherapy sensitive relapsed acute leukemia or lymphoma, and for some non-malignant diseases, such as BM failure in Fanconi anemia [7–11]. Under physiological conditions, few HSCs circulate in peripheral blood (PB) [12]. The induced movement of HSCs from BM into PB is termed HSC mobilization. Mobilized PB stem cells have become a preferred stem cell source for autologous transplantation, and are widely used for allogeneic stem cell transplantation [13–16].

G-CSF is the standard of care in mobilizing HSCs for transplantation. However, broad inter-individual variability exists in response to this mobilization regimen, and poor HSC mobilization in response to G-CSF is often found in patients who have been treated for cancer or who have genetic disorders [8, 9, 17]. AMD3100, a CXCR4 antagonist, has been used alone or in combination with G-SCF to mobilize stem cells [18–21]. A recent study found that AMD3100 mobilizes a different set of HSCs than G-CSF [19], raising the possibility that the PB stem cells collected by these two regimens may have different properties. Indeed, a more recent study using AMD3100 in combination with CXCR2 agonist, GROβ shows a preferential trafficking of stem cells with high-engraftment efficiency resulted from synergistic signaling on neutrophils [22]. Hence, development of new methods to mobilize HSCs, alone or in combination with existing regimens, will continue to have therapeutic value to further improve transplant outcomes.

The Rho family small GTPase Cdc42 regulates actin organization, cell–cell and cell-extracellular matrix adhesions, and directional movement in many cell types [23, 24]. In HSCs, Cdc42 integrates signals from multiple cell surface receptors—including c-Kit, CXCR4, and β1 integrin—to regulate cytoskeleton dynamics that affects cell polarity, adhesion and migration properties in normal HSC maintenance [25–27]. Unlike the related Rac1 and Rac2 GTPases [28, 29], Cdc42 is not required for HSC survival or proliferation but is essential in mediating HSC homing, lodging, and retention in the bone marrow niche [25]. Studies of a Cdc42 gain-of-activity using the Cdc42GAP knockout mouse model have revealed that constitutively elevated Cdc42 activity similarly causes defective adhesion and migration leading to decreased engraftment of HSCs [30], suggesting that a precise regulation of Cdc42 activity is required for retaining HSCs in the BM niche. The current studies are aimed to develop a new concept, i.e., pharmacologic targeting of Cdc42 is of therapeutic value for effective mobilization of LT-HSCs thus benefiting stem cell harvest, in preclinical mouse models. Our work not only stringently examines the effect of Cdc42

targeting in HSC mobilization, but also validates a lead small molecule inhibitor specific for Cdc42 activity that could be broadly useful to study biological and pathological roles of Cdc42 in blood and other tissue cells.

Materials and methods

Animals

C57Bl/6 and congenic BoyJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in the animal barrier facility at the Cincinnati Children's Hospital Medical Center. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center prior to study initiation (IACUC protocol # 2013–0159).

CASIN

CASIN and its inactive analog (referred to Peterson et al., as Pirll-related compound 2 and 7, respectively) [31] were obtained from Chembridge Corporation, and purified to >99% by high-performance liquid chromatography. For in vitro studies, CASIN (containing 0.02–0.2% DMSO) was used at 2–20 μM in IMDM. For in vivo experiments, CASIN was dissolved in PBS with 15% ethanol, and administered by IP (2.4 mg/kg) or IV (1.2 mg/kg) injection. Separately, C57Bl/6 mice were treated with AMD3100 at 5 mg/kg by IP.

Flow cytometry

HSPCs were immunophenotyped using FACS analysis with the phenotypic antibodies IL-7Rα-APC-cy7, c-Kit-APC, Sca-1-PE, CD34⁻FITC, FcRII/III (CD16/32)-PE-cy7, as well as biotinylated lineage-depletion cocktail labeled with the secondary antibody Percp-cy5-streptividin. The sub-compartments of HSPCs, including LT-HSC and ST-HSC were then quantified accordingly. Moreover, LT-HSCs are also defined by FCAS analysis using antibodies of LSK as above together with CD150-PE-cy7, CD48/41-FITC. Blood cell differentiation was determined by the flow cytometry after staining with CD3, B220, CD11b, Gr1, and Ter119. Cell cycle and apoptosis were determined by flow cytometry after BrdU labeling and and/or annexinV and 7AAD staining.

CFU determination of HPC mobilization

C57Bl/6 mice were mobilized with CASIN (1.2 mg/kg, IV), and progenitor cell numbers in PB were determined at 0.5, 1, 2, and 4 h post CASIN injection. At each time point, 200 μl of PB was added to 4 ml of cytokine-containing methylcellulose (Stem Cell Technologies, Vancouver, Canada) and plated in triplicate in 35 mm-gridded dishes. Colonies were enumerated 7 days post plating. Separately, C57Bl/6 mice were treated with AMD3100 at 5 mg/kg by IP or with huG-CSF (Amgen) at 12.5 μg/kg in PBS/0.1% BSA by IP at 100 μg/kg per day once a day for 5 days and animals were analyzed on day 6. In the combinatory treatment, CASIN was administrated by IV (1.2 mg/kg) injection on day 6 of G-CSF regimen or together with AMD3100 and PB samples were analyzed 2 h post CASIN treatment.

Radioprotection assay, serial transplantation, and chimerism analysis

Groups of donor CD45.2 C57B1/6 mice ($n = 4$) were treated by IV injection of CASIN and vehicle control and IP injection of AMD3100. Mobilized PB were collected 2 h after by cardiac puncture. Recipient CD45.1 BoyJ mice ($n = 4$) were lethally irradiated (11.75 Gy) prior to transplantation and injected intravenously with PBMC from 500 μl PB. Morbidity was examined everyday by 2 weeks and weekly until month 4 when the secondary transplantation was performed using 5×10^6 BM cells of the primary recipients. Chimerism was analyzed by the flow cytometry on total, $CD3^+$, $B220^+$, $Gr1^+$, $Ter119^+$, and $LSKCD34^-$ population of PB and BM cells.

For detailed experimental procedures, see Supplemental Methods.

Results

Cdc42 activity-specific inhibitor, CASIN, specifically inhibits Cdc42 guanine nucleotide exchange activity

In search of a Cdc42-specific small molecule inhibitor, we examined compounds, including Pirl that show inhibitory activity in an in vitro Xenopus oocyte lysate assay of actin polymerization induced by Phosphatidylinositol 4,5-bisphosphate (PIP2) [31], which may directly involve the Cdc42-WASP (Wiskott-Aldrich syndrome protein) signaling cascade. An analog of Pirl, the lead chemical for F-actin inhibition reported by Peterson [31], which we term **C**dc42 **a**ctivity-**s**pecific **in**hibitor (CASIN; Fig. 1a), was found to confer the most active Cdc42 binding activity in an in vitro binding assay, in which purified proteins were labeled with fluorescence dye and microscale thermophoresis were measured by a NanoTemper Monilith Instrument [32], in comparison with Pirl1 and another inactive analog (Fig. 1b, S1). CASIN directly interacted with purified Cdc42 with an affinity of 332 ± 101 nM Kd (Fig. 1b) and did not bind to related Rho GTPase RhoA, Rac1, RhoJ, RhoU, or RhoV, nor the Cdc42 guanine nucleotide exchange factor (GEF) intersectin (Fig. 1b and S2A, S2B). In the GEF reaction of Cdc42 catalyzed by the DH-PH module of intersectin GEF, CASIN showed a dose-dependent inhibition of GEF-induced dissociation of GDP from Cdc42; whereas in similar experiments CASIN did not affect RhoA guanine nucleotide exchange reaction catalyzed by the RhoA-specific GEF, LARG (Fig. 1c). These data indicate that CASIN binds specifically to Cdc42-GDP. When CASIN were examined in fibroblast cells, we found that it inhibited bradykinin-induced F-actin morphologic changes of wild-type (WT) mouse embryonic fibroblast (MEF) cells but showed no effect on *Cdc42*$^{-/-}$ cells that are resistant to bradykinin stimulation (Figure S3). Thus, CASIN can directly bind to Cdc42 and specifically inhibit Cdc42 GEF reaction.

CASIN inhibits Cdc42 activity, F-actin polymerization, and polarization of blood progenitors

To examine the effect of CASIN in inhibiting Cdc42 activity in primary hematopoietic cells, murine low-density bone marrow (LDBM) cells were treated with CASIN, and the effect on endogenous Cdc42-GTP species was determined by an effector-domain pull-down assay [27, 29]. As shown in Fig. 2a, 5 μM CASIN could suppress Cdc42-GTP formation, but not Rac1 activity, activated by stem cell factor (SCF), Stromal cell-derived factor 1α (SDF-1α)

or fibronectin. Further, CASIN caused a dose-dependent inhibition of SDF-1α-induced Cdc42 activation without affecting the activity of the closely related Rac1 or RhoA, and removal of CASIN by a wash of the cells restored Cdc42 responsiveness to SDF-1α (Fig. 2b; data not shown). CASIN also induced a dose-dependent suppression of polarized distribution of Cdc42 and tubulin in blood progenitor cells (Fig. 2c). Similar effects of CASIN were observed in F-actin polymerization and polarity assays of primitive long-term HSCs (LT-HSCs; Fig. 2d, S4). CASIN, on the other hand, did not alter the maturation of primary hematopoietic progenitor cells (HPCs) into multi-lineage progenitors detected by colony-forming assays (Fig. 2e). These results suggest that CASIN can selectively and reversibly inhibit Cdc42 activity regulated by cytokine, chemokine, and adhesion molecules in HPCs without affecting HPC differentiation.

CASIN dose-dependently suppresses adhesion and migration of wild-type blood progenitor cells but has no effect on *Cdc42*$^{-/-}$ HPCs

To further ascertain the functional specificity of CASIN, we next examined the effect of CASIN on the Cdc42-regulated cellular functions in WT and *Cdc42*$^{-/-}$ HPCs. In WT but not *Cdc42*$^{-/-}$ Lin^{-} BM cells (enriched for HPCs), CASIN was effective in suppressing basal and SDF-1α stimulated Cdc42 activity but did not affect Rac1-GTP formation (Fig. 3a), consistent with our observation that CASIN directly and specifically interacts with purified Cdc42 but not other Rho GTPases, including Rac1 and RhoA (Fig. 1b, S2). CASIN treatment of Lin^{-} BM cells led to a dose-dependent inhibition of adhesion to fibronectin by colony-forming cells, and impairment of directional migration toward a SDF-1α gradient by colony-forming unit (CFU) cells, mimicking that of *Cdc42* gene deletion when it was used at a higher dosage (Fig. 3b, c). While CASIN dose-dependently suppressed adhesion and directional migration activities of WT Lin^{-} BM cells to the extent of *Cdc42*$^{-/-}$ cells, no additive cellular effects of CASIN in these assays were detected upon CASIN treatment of *Cdc42*$^{-/-}$ Lin^{-} BM cells (Fig. 3b, c). More importantly, CASIN treatment did not affect proliferation of WT or *Cdc42*$^{-/-}$ LDBM cells (Fig. 3d), allowing *Cdc42*$^{-/-}$ cells to retain a higher proliferative activity than WT cells as previously observed [25]. In primary HPCs, CASIN inhibited several known Cdc42 downstream-signaling events, including phosphor-PAK1, phosphor-WASP, and phosphor-aPKC, similar to that by *Cdc42* gene deletion (Fig. 3e). Together, these results indicate that CASIN suppresses adhesion and migration but does not affect the proliferation of HPCs. Importantly, CASIN does not appear to have detectable effects on *Cdc42*$^{-/-}$ bone marrow cells.

CASIN specifically inhibits Cdc42 activity and Cdc42-mediated signaling in HSPCs

To probe for the molecular mechanism of CASIN action, we transduced hematopoietic stem and progenitor cells (HSPCs) from *Cdc42*$^{-/-}$ mice with lentiviral vector expressing a constitutive active Cdc42 mutant, Q61L (Cdc42-Q61L), and found that CASIN was incapable of suppressing F-actin polymerization in *Cdc42*$^{-/-}$ HSPCs expressing the Cdc42-Q61L mutant protein (Fig. 4a), suggesting that CASIN acts at the activation site but not the effector site of Cdc42. We also carried out a simulation of CASIN binding to the Cdc42 three-dimensional (3D) structure and found that CASIN can interact with a surface groove of Cdc42 that is required for interaction with GEFs such as DOCK180 (Fig. 4b). Further, mutational analysis of surface residues of Cdc42 reveals that Cdc42-M45E mutation at the

GEF action site resulted in a loss of the binding activity to CASIN in vitro (Fig. 4c) and the unresponsiveness to CASIN in cells where endogenous WT Cdc42 was readily inhibited by CASIN (Fig. 4d). Collectively, these results indicate that CASIN selectively inhibits Cdc42 activity and Cdc42-mediated signaling in HSPCs.

CASIN induces mobilization of HSCs in mice

Adult HSCs reside primarily in the BM, with only a small faction in the peripheral blood (PB) under normal physiologic conditions [3, 12, 33]. Previous gene targeting experiments in mice have found that Cdc42 knockout in mice results in massive mobilization of HSCs from the BM to the peripheral [25]. To examine whether CASIN could mobilize HSCs in mice, we applied a protocol of intravenous (IV) injection of CASIN to C57Bl/6 mice. Pharmacokinetic analysis indicates that CASIN concentration in the serum of injected animals was quickly reduced from 20 μM to less than the effective concentration of 0.5 μM in ~30 min (Fig. 5a, S5), suggesting that the effect of CASIN is transient. IV injection of CASIN in mice resulted in a marked inhibition of Cdc42 activity in LDBM hematopoietic cells in vivo (Fig. 5b). Colony-forming unit in culture (CFU-C) assay showed that CASIN was able to induce a fourfold increase of progenitor activities in the peripheral blood (PB) compared to controls 2 h after CASIN injection (Fig. 5c). Concomitant with the CASIN-induced increase in colony-forming activity in PB, a significant increase in progenitor activity was observed in spleen and liver of the mice (Fig. 5d). Consistent with the lack of effect on *Cdc42*$^{-/-}$ cells in vitro, the effect of CASIN in mobilizing HPCs appears to be specific through Cdc42, as CASIN administration showed no additive effect on mobilized CFU-C activity in *Cdc42*$^{-/-}$ mice (Fig. 5e).

To examine potential toxicity of transient CASIN treatment, both long-term IV and IP injection protocols of CASIN into mice in a broad range of dosages (0.1–5 mg/kg) were tested. No detectable toxicity on multi-lineage blood differentiation were observed by either H&E staining or marker FACS analysis of the BM, liver, spleen, and PB up to a period of 6 months of daily injection (Figure S6 and data not shown). Further, CASIN does not appear to alter the survival or cell cycle status (Figure S7 and data not shown) and mobilizes BM HSPCs that confer all different lineages of cells (Fig. 5f) in the treated mice. Taken together, these results demonstrate that CASIN is safe and effective in mobilizing blood progenitors in vivo.

CASIN-mobilized HSCs are superior in functionality to those mobilized by AMD3100

Cytokine granulocyte colony-stimulating factor, G-CSF and a CXCR4 antagonist, AMD3100 have become the standard of care in mobilizing HSCs for transplantation. AMD3100 has been clinically used alone or in combination with G-SCF to mobilize stem cells [18–21]. To determine the potential applicability of CASIN in preclinical settings, we then attempted to compare the mobilization capacity of CASIN, over AMD3100 or G-CSF. In a progenitor colony-forming unit (CFU) activity assay, we found that all 3 agents were able to mobilize progenitor cells from BM to peripheral blood, with G-CSF treated mice showing the greatest CFU activity in PB (Fig. 6a). Since G-CSF has non-responders and potential issue on growth stimulation in the clinics, and since G-CSF administration requires a distinct 5-day injection protocol, we decided to focus on a comparison of CASIN with

AMD3100, a small molecule antagonist of CXCR4 which shares signaling with Cdc42 and requires only a single injection [34]. We found that CASIN administration led to a ~6-fold increase of phenotypically defined LT-HSCs (LSKCD150^{+}CD48^{-}CD41^{-}) in PB 2 h after IV injection, whereas AMD3100 caused a ~5-fold increase of mobilization of phenotypic HSCs (Fig. 6b). More importantly, we sought to determine the functionality of the HSCs mobilized by CASIN or AMD3100. To this end, PB harvested from the vehicle, CASIN or AMD3100-treated mice were analyzed in a transplant assay to determine radioprotection by transplanted HSCs in lethally irradiated recipient mice. Both the CASIN and AMD3100 mobilized HSPCs were found capable of rescuing lethally irradiated mice, while transplant of PB from vehicle-treated mice resulted in death of the recipients in 4 weeks (data not shown). The primary recipients of transplanted PB from CASIN or AMD3100 mobilized mice showed comparable, over 80% donor-derived blood cells in PB and BM in multiple lineages (Fig. 6c and Figures S8A, S8B). Remarkably, the CASIN-mobilized HSCs reconstituted over 90% of the LSKCD34^{-} compartment in the primary recipients (Fig. 6d), indicating a long-term reconstitution potential of CASIN-mobilized HSCs. In addition, expression of CD26 in LSK population of BM cells or serum SDF-1α level did not change after CASIN injection (Figures S8C, S8D), suggesting that CASIN does not affect CD26-mediated effects like G-CSF or serum SDF-1α content [35, 36]. Although CASIN displayed only a slight improvement in mobilization capacity for phenotypic HSCs over AMD3100 (sixfold vs fivefold; Fig. 6b), secondary transplant of the BM cells from primary recipients showed that CASIN-mobilized HSCs were superior in quality to those mobilized by AMD3100, as they were able to maintain over 90% PB or BM chimerism of various lineages in the secondary recipients 6 months post transplant, whereas AMD3100-mobilized PB resulted in only ~15% and 35% chimerism in the BM and PB, respectively (Fig. 6e, f). When CASIN was applied to mice in combination with AMD3100 or G-CSF, an additive effect in mobilizing HSPCs to PB was detected by CFU assays (Fig. 6g, h). Taken together, these data show that pharmacological inhibition of Cdc42 by CASIN leads to a transient mobilization of LT-HSCs with a superior quality.

Discussion

HSC mobilization is a standard procedure in BM transplantation for the treatment of many hematologic diseases, including BM failure syndromes and leukemia [9–11]. Current clinical HSC mobilization utilizes only G-CSF and less frequently AMD3100 as mobilization agents with limitations in efficacy and patient application [9, 17, 19, 21]. There is a great need for improved HSC mobilization regimens. We report here that the chemical inhibitor CASIN specifically and reversibly inhibits Cdc42 activity, which can transiently mobilize long-term engraftable HSCs. There are several findings that highlight the significance of our study: (1) CASIN binds specifically to Cdc42 and competitively interferes with its guanine nucleotide exchange activity; (2) CASIN reversibly inhibits Cdc42 activity by suppressing actin polymerization, adhesion, and directional migration; (3) CASIN transiently mimics Cdc42 knockout phenotype in inducing HSPC egress from mouse BM; (4) CASIN administration to mice mobilizes HSCs with better long-term reconstitution potential than those mobilized by AMD3100.

Core values of a chemical inhibitor or probe include its specificity and reversibility, which are the causal factors for side effects or toxicity. We demonstrated that CASIN can specifically and transiently inhibit Cdc42 activity without notable effects on the closely related Rac1 or RhoA. To date, several Cdc42 inhibitors have been reported. The first identified small molecule ML141 (CID-2950007) has been shown to inhibit nucleotide binding of Cdc42 GTPase; however, ML141 also exhibits low-micromolar potency and selectivity against other members of the Rho family of GTPases, including Rac1, Rab2, and Rab7 [37, 38]. In addition, recent studies show that ML141 fails to substantially reduce Cdc42 activity and alleviate cell migration [39]. The second Cdc42 inhibitor AZA1 suppresses both Cdc42 and Rac1 activities in a dose-dependent manner [40]. Another small molecule, ZCL278 specifically targets Cdc42-intersectin interaction and inhibits Cdc42-mediated cellular processes, but its action is not reversible [41]. Finally an inhibitor targeting the activated Cdc42-associated kinase 2 (ACK-2) not only binds Cdc42 in the GTP-bound form but also interacts with clathrin and influences clathrin assembly [42, 43]. In the present study, we show that CASIN specifically binds to purified Cdc42 protein but does not bind to other Rho GTPases, including RhoA, Rac1, RhoJ, RhoU or RhoV, nor the Cdc42 guanine nucleotide exchange factor (GEF) intersectin (Fig. 1b, c and S2). Furthermore, CASIN inhibits Bradykinin-induced filopodia formation mediated by Cdc42 in WT cells but shows no effect on *Cdc42*$^{-/-}$ cells (Figure S3). More importantly, CASIN does not show any detectable effects on *Cdc42*$^{-/-}$ HSPCs in multiple functional assays, and CASIN administration to mice mimics Cdc42 knockout to transiently mobilize HSCs and shows no detectable toxicity (Figure S6, S7) nor effects on multi-lineage blood differentiation (Fig S8). Since CASIN has a rather shorter lifetime by the i.v. injection protocol (Figures S5) and since Cdc42 activity is transiently and reversibly suppressed by CASIN, CASIN can be repeatedly administered to transplant recipients without causing insensitivity. Therefore, the stringent criteria in specificity and reversibility demonstrated by CASIN distinguishes it from other Cdc42 chemical inhibitors.

Another intriguing finding of our study is that CASIN appears superior to AMD3100 in producing functional long-term HSCs in the mouse transplantation model. Previous studies have demonstrated that while Cdc42 inhibitor ML141, which inhibits nucleotide binding of Cdc42 alone only triggers modest HSPC mobilization, combination of G-CSF and ML141 significantly increases HSPC counts and colony-forming units in PB, as compared to mice treated with G-CSF alone [44]. This might be, at least in part, due to non-specific nature of ML141. Our present results demonstrate that CASIN specifically inhibits Cdc42 activity through inhibiting Cdc42 guanine nucleotide exchange activity. Although, the PB containing mobilized HSCs from both CASIN- and AMD3100-treated donor mice were capable of rescuing the lethally irradiated primary recipient mice, the CASIN-mobilized PB was able to maintain over 90% PB or BM chimerism of various lineages, including the stem cell-enriched LSK compartment, in the secondary recipients 6 months post transplant. In comparison, the AMD3100-mobilized PB resulted in only ~15% and 35% chimerism in the BM and PB, respectively, and almost undetectable donor LSK cell population in the BM of the secondary recipients (Fig. 6). It should be noted that our study compared the effects of CASIN to those with a single dose of AMD3100. In this context, further detailed optimization of CASIN mobilization protocol compared with the AMD3100 multi-dosage

protocol may reveal additional differences and similarities. Our results with CASIN in mobilizing functional HSCs anchors a recent study reporting that a CXCR2 agonist, GROβ in combination with AMD3100 are more effective than G-CSF in mobilizing engraftable HSCs in mice [22]. However, GROβ + AMD3100 shows disadvantage in mobilizing total hematopoietic progenitor (LSK) cells and phenotypic (SLAM LSK) HSCs compared to G-CSF [22]. Thus, it seems that CASIN, acting as a single agent, can be as potent as the combination regimen of GROβ + AMD3100 in mobilizing functional hematopoietic stem and progenitor cells from the BM to the peripheral.

Our present study demonstrates that CASIN is safe and effective in mobilizing blood progenitors in vivo without detectable toxicity or affecting multi-lineage blood cell differentiation (Fig S6). In addition to its application in mouse model, we recently show that CASIN effectively and transiently mobilizes xenografted human $CD34^+$ hematopoietic progenitor cells to the PB 1 h after injection [45]. Furthermore, conditioning with CASIN significantly facilitates engraftment of human blood progenitors in a transplant model without any myeloablation [45]. Although further improvement on its potency and better understanding the benefits of its short half-life (reversibility) in vivo are needed, we believe that CASIN may represent a safe and improved pre-conditioning agent for future human hematopoietic stem cell transplantation.

A major challenge for successful BM transplantation is effective mobilization and harvesting of donor HSCs with sufficient number and functional quality. Novel methods of HSC mobilization and improved engraftment are needed for treatment of blood diseases through BM transplantation, especially for BM failure and leukemia patients who have limited stem cell numbers and are intolerable to intensive pre-conditioning regimens. In this report, we provide evidence that CASIN specifically and transiently inhibits Cdc42 activity and acts as a single agent to effectively mobilizing phenotypic and functional HSCs to the peripheral. Since our previous genetic studies have established that Cdc42 is essential for the retention of HSCs in the BM niche [25, 26], CASIN may prove to be a new method for blood stem cell collect and transplantation that may significantly impact on future BM transplantation. Specifically, CASIN would improve both sides of the transplant practice—providing a new way to mobilize HSCs from donors and improving engraftment efficiency in recipients without increasing treatment-related toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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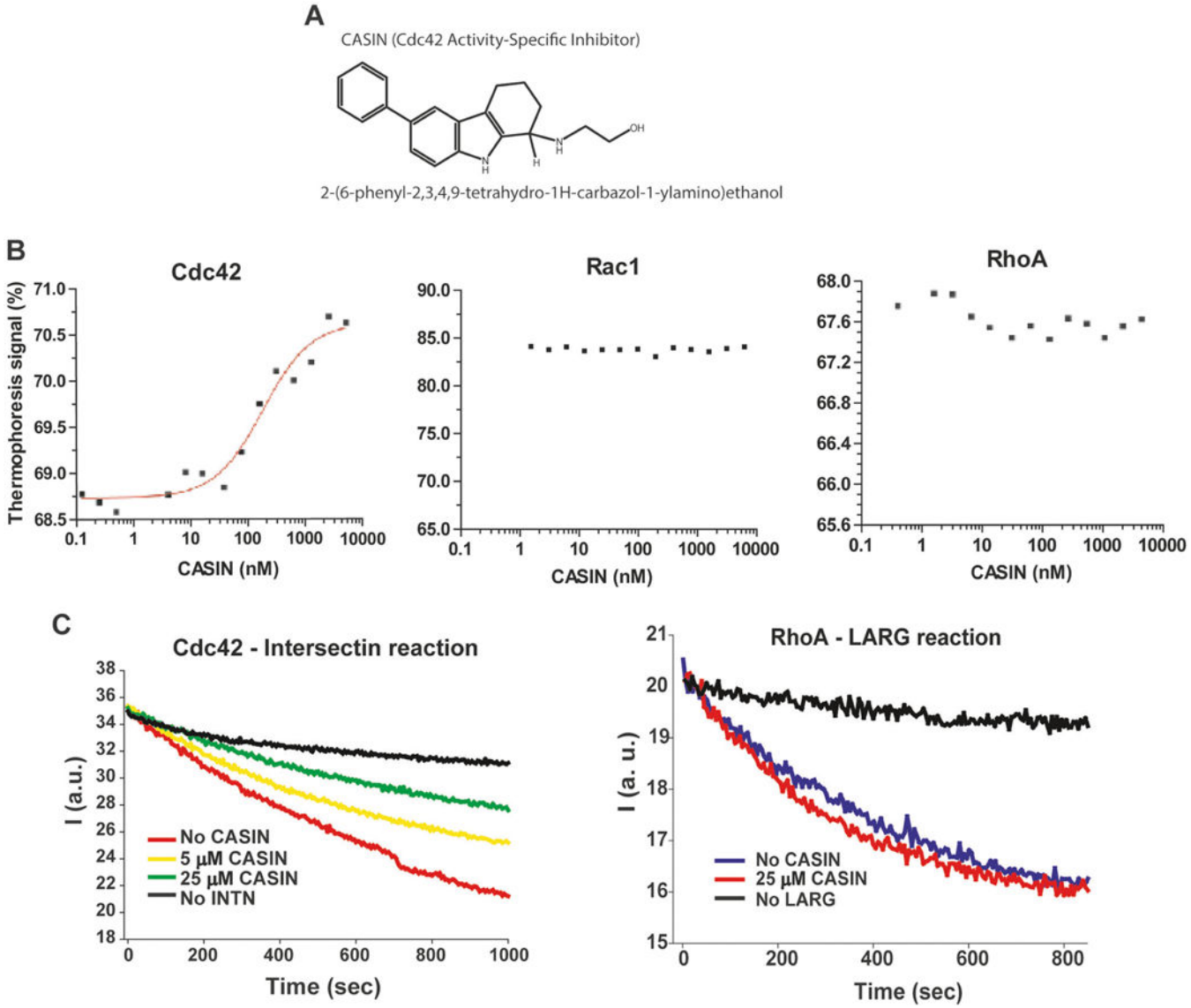

Fig. 1.

Chemical and genetic evidence that CASIN specifically and reversibly inhibits Cdc42 activity. **a** Chemical structure of CASIN. **b** Microscale thermophoresis measurement of CASIN binding to purified Cdc42 protein. Fluorescently labeled Cdc42, Rac1, or RhoA (200 nM) was subjected to titration of increasing concentrations of CASIN. The relative changes in thermophoresis were traced. Results are representative of at least three independent experiments. **c** Guanine nucleotide exchange reaction was performed using fluorescent BODIPY FL-GDP bound to Cdc42 (50 nM), catalyzed by intersectin (INTN; 9 nM) (left) or using fluorescent BODIPY FL-GDP bound to RhoA (50 nM), catalyzed by LARG (10 nM) (right) in the presence or absence of CASIN (5 or 25 µM). Results are representative of at least three independent experiments

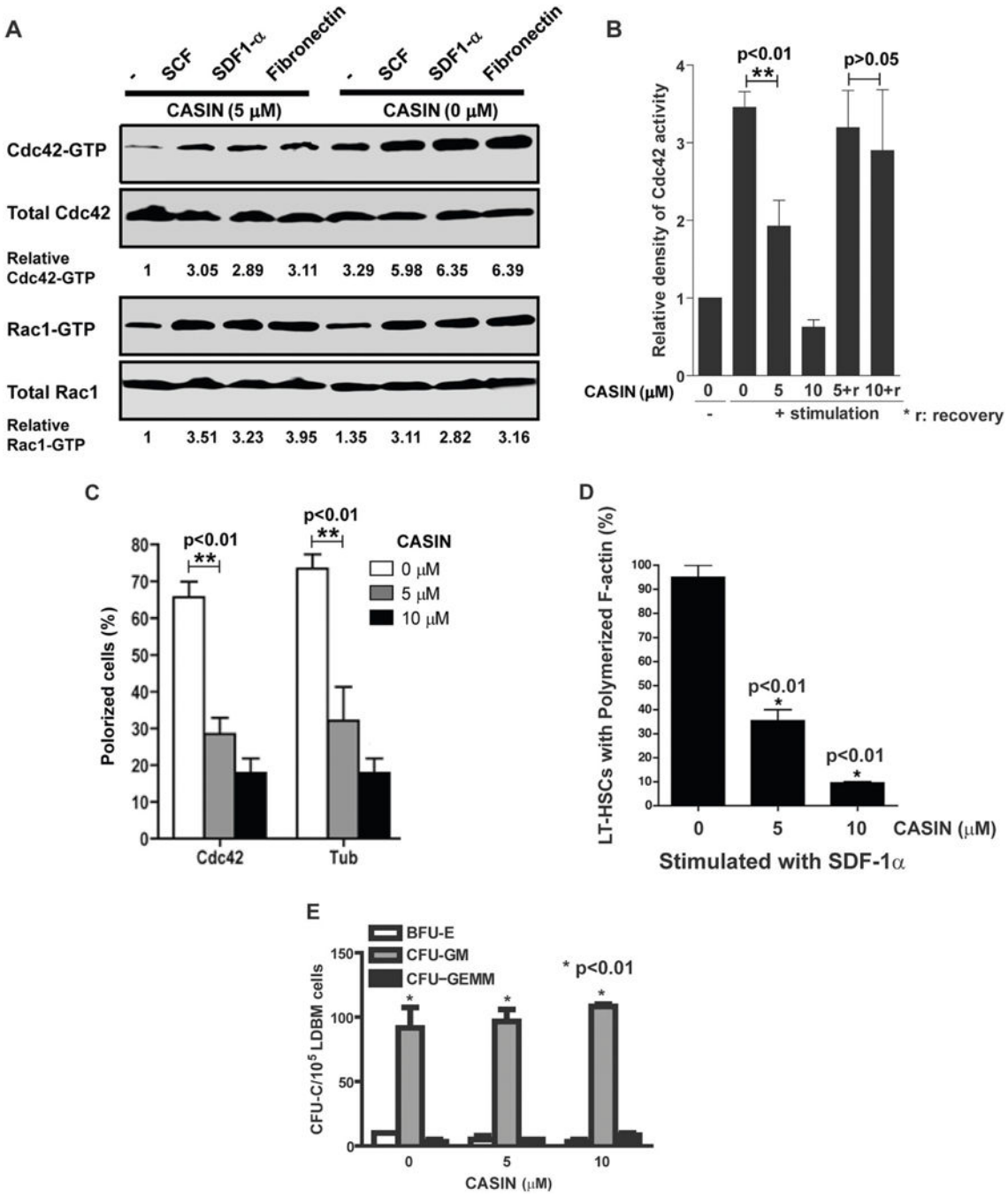

Fig. 2.

CASIN inhibits Cdc42 activity, F-actin polymerization and cell polarity of hematopoietic cells. **a** Isolated low-density bone marrow (LDBM) cells from WT C57Bl/6 mice were cultured overnight in a medium without cytokines. The effect of CASIN on Cdc42 activity as determined by GST-Pak1 effector-domain pull-down assays in the LDBM cells stimulated with or without SDF-1α (100 ng/ml, 15 min), SCF (20 ng/ml, 15 min), or fibronectin (100 nM, 15 min) in the presence of CASIN (5 μM). The quantification of the relative levels of Cdc42-GTP or Rac1-GTP to Cdc42 or Rac1, respectively, was performed by densitometry

using the NIH J Image software. **b** Quantification of Cdc42 activity determined by Cdc42 effector-domain pull-down assays in LDBM cells with or without SDF-1α stimulation (100 ng/ml, 15 min) and/or CASIN treatment (5–10 μM). After CASIN treatment, some samples were washed once with PBS, and then challenged with SDF-1α. r: recovery after the wash. Error bars represent standard deviations. Results are means ± SD from three independent experiments. **c** Effect of CASIN on the polarity of blood progenitors. CASIN at indicated increasing concentrations was added to cultured $Lin^{-}Kit^{+}$ progenitors of WT C57Bl/6 mice, and the polarized distributions of endogenous Cdc42 and Tubulin were quantified after immunofluorescent staining for Cdc42 and Tubulin, respectively. Results are representative of at least three independent experiments. **d** Effects of CASIN on F-actin polymerization in long-term (LT)-HSCs. Freshly isolated $Lin^{-}Sca1^{+}Kit^{+}CD34^{-}Flk2^{-}$LT-HSCs from WT C57Bl/6 mice were incubated for 16 h in medium without growth factors in the present or absence of the indicated concentrations of CASIN. The cells were then stimulated with SDF-1α (100 ng/ml) for 15 min before fixation. Immunofluorescence staining of F-actin by Fluorescein-phalloidin and Nuclei with DAPI were carried out. Cells with polymerized cortical F-actin distribution were quantified. n = 20–25 cells in each group. Results are representative of at least three independent experiments. **e** CASIN does not alter the BFU-E, CFU-GM, or CFU-GEMM activity of WT C57Bl/6 LDBM cells. LDMCs were treated with different doses of CASIN for 2 h followed by CFU assay using methycellulose medium supplemented with rmSCF, rmIL-3, rhIL-6, and ehEPO in triplicate in 35 mm-gridded dishes. Colonies were enumerated on day 7. Results are representative of at least three independent experiments

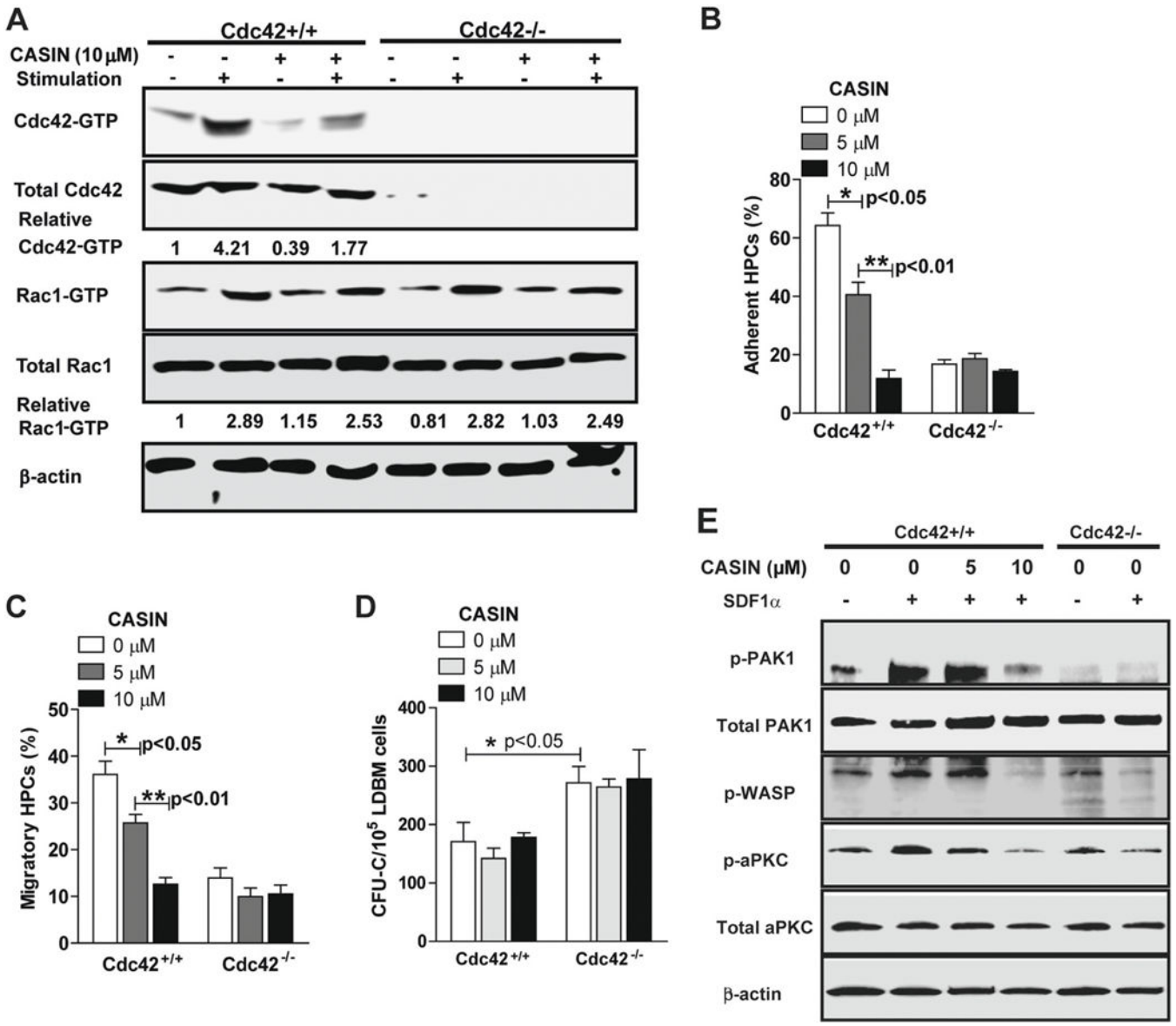

Fig. 3.

CASIN dose-dependently suppresses adhesion and migration of blood progenitors. **a** Effector domain pull-down assay for Cdc42 and Rac1 activities in *Cdc42*$^{+/+}$ and *Cdc42*$^{-/-}$ murine LDBM cells with or without SDF-1α stimulation (100 ng/ml, 15 min) and/or CASIN treatment (10 μM). The relative Cdc42-GTP and Rac1-GTP levels in each sample were normalized to that of the *Cdc42*$^{+/+}$ cells without stimulation and CASIN treatment. Results are representative of at least three independent experiments. **b**, **c** Effects of CASIN on adhesion (to fibronectin fragment CH296) (**b**) and migration (toward an SDF-1α gradient) (**c**) of murine BM-derived colony-forming progenitors from *Cdc42*$^{+/+}$ or *Cdc42*$^{-/-}$ mice (n = 3). Results are representative of at least three independent experiments. **d** CASIN does not affect differentiation and proliferation of progenitor activities of LDBM cells. Comparison of the effects of CASIN on CFU-C (mix) activities between *Cdc42*$^{+/+}$ and *Cdc42*$^{-/-}$ LDBM cells. CASIN at 0, 5, 10 μM concentrations were examined in each condition. Results are

representative of at least three independent experiments. **e** Immunoblots of Cdc42 effectors p-PAK1, p-WASP, and p-aPKC in CASIN-treated (5 or 10 μM) WT Lin^- cells, compared with *Cdc42*$^{-/-}$ Lin^- cells, after SDF-1α stimulation (100 ng/ml, 15 min). All error bars represent standard deviations. Results are representative of at least three independent experiments

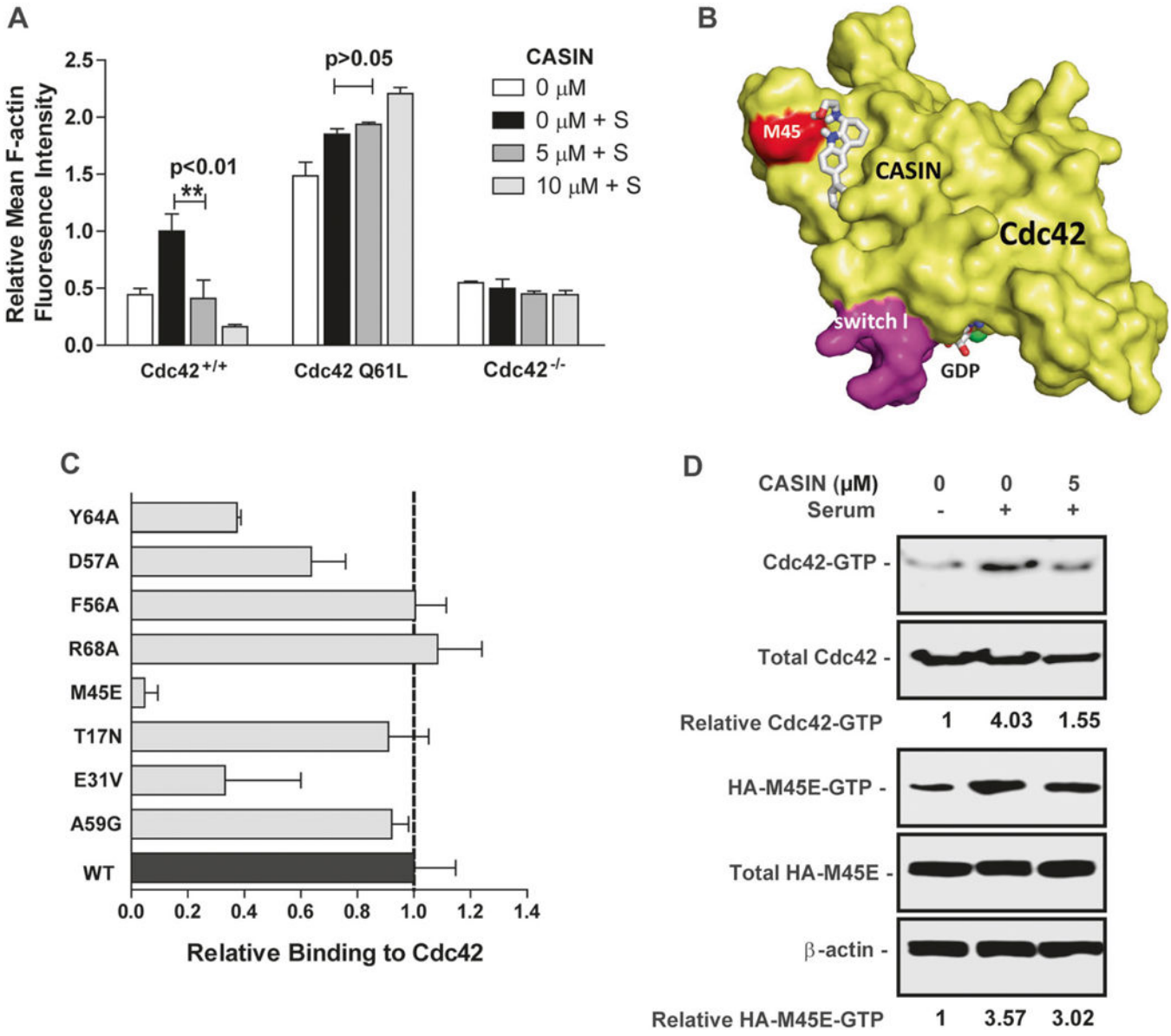

Fig. 4.

CASIN specifically inhibits Cdc42 activity and Cdc42-mediated signaling in HSPCs. **a** SDF-1α-induced F-actin polymerization analyzed by FACS analysis ($n = 3$) in WT murine Lin^- cells with or without CASIN treatment (0, 5, 10 μM), in comparison to that of Q61L-transduced or *Cdc42*$^{-/-}$ cells. S SDF-1α stimulation at 100 ng/ml for 15 min. Results are representative of at least three independent experiments. **b** CASIN binding to Cdc42 was simulated by a structural modeling with the critical Met45 residue involved in CASIN binding, which interfaces with DOCK GEF binding surface as indicated. **c** Relative binding of CASIN to Cdc42 point mutants in comparison to wild-type Cdc42. Relative binding affinity was normalized to that of wild-type Cdc42. Results are representative of at least three independent experiments. **d** Representative immunoblots of effector domain pull-down assays for Cdc42 activity in 293T cells transfected with M45E Cdc42, with or without serum

stimulation (20% FBS, 15 min) and/or CASIN treatment (5 μM). The endogenous WT Cdc42-GTP level was measured in parallel to M45E-GTP in the same samples. Results are representative of at least three independent experiments

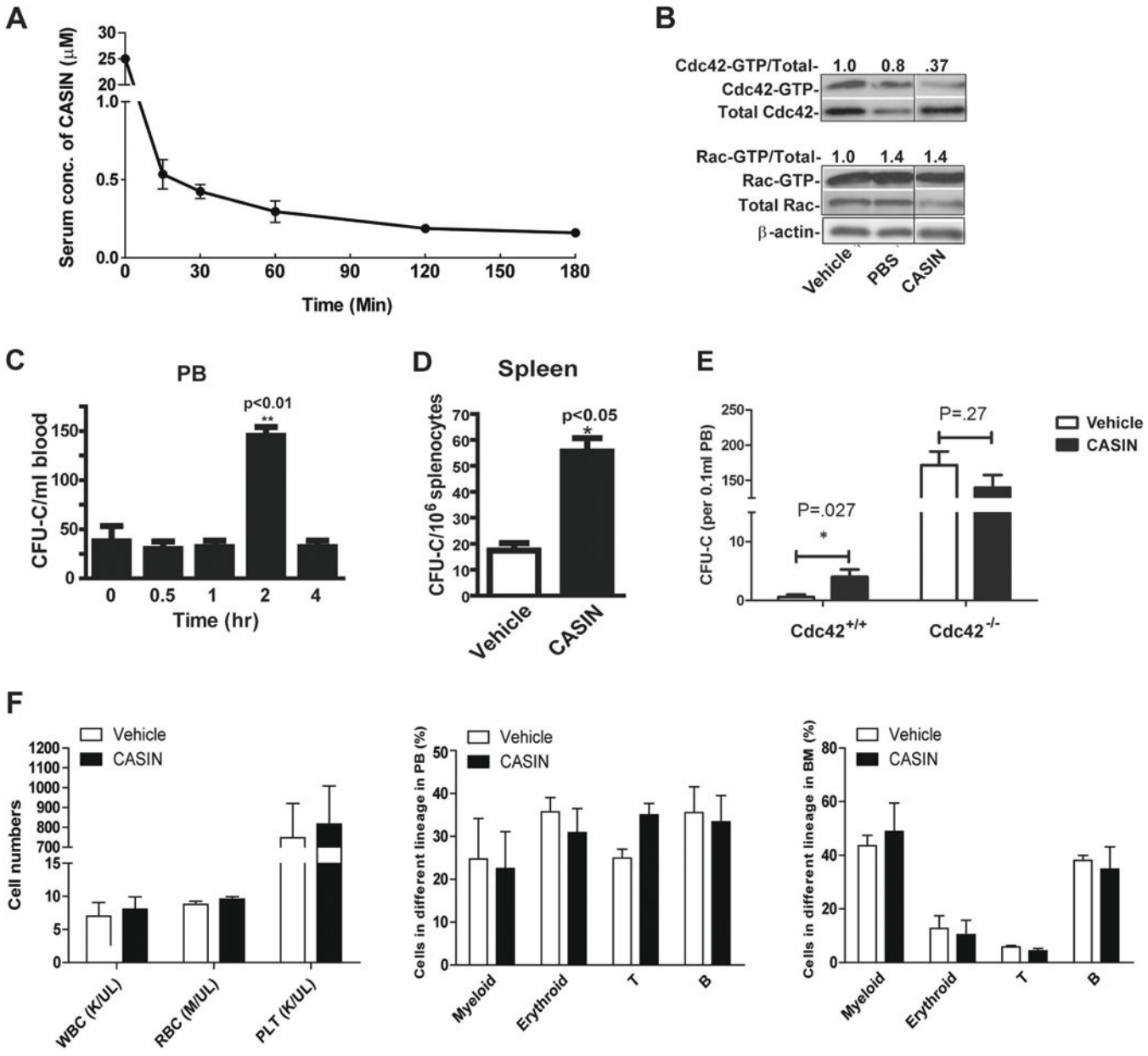

Fig. 5.

CASIN phenocopies *Cdc42* gene deletion to induce mobilization of long-term HSCs. **a** Pharmacokinetics of CASIN in mouse sera determined by HPLC-ESI-MS/MS after CASIN administration (1.2 mg/Kg) by IV. Results are representative of at least three independent experiments. **b** Representative immunoblots of effector-domain pull-down assays for Cdc42 and Rac1 activities in LDBM cells of CASIN-or vehicle-treated mice. LDBMCs were isolated from the mice 2 h after CASIN administration (1.2 mg/ Kg, IV) followed by Cdc42 effector pull-down assay with or without PBS wash. Results are representative of at least three independent experiments. **c** Mobilization of hematopoietic progenitor CFU activities in peripheral blood post-CASIN injection (1.2 mg/kg) *(n* = 6, 3, 4, 7, and 4); ***P* < 0.01, basal vs 2 h. **d** CFU progenitor activities of splenocytes 2 h after CASIN treatment (1.2 mg/kg,

IV) (n = 3). Results are representative of at least three independent experiments. **e** CASIN induces mobilization of colony-forming progenitors in WT mice but does not show additive effects in *Cdc42*$^{-/-}$ mice. Peripheral blood from WT and *Cdc42*$^{-/-}$ mice (5 days after polyI:C injections) that were subjected to either vehicle or CASIN treatment, were collected 2 h after the vehicle or CASIN IV administration. The colony-forming activities of the PB cells were assayed. CASIN did not cause a detectable effect in the *Cdc42*$^{-/-}$ mice under conditions where WT mice were responsive. Results are representative of at least three independent experiments. **f** CASIN mobilizes different lineages of cells. Effects of CASIN on blood cell mobilization were determined by flow cytometry and complete blood count 2 h after CASIN administration (1.2 mg/Kg, IV) in comparison to controls (n > 6). Left: Numbers of WBCs, RBCs, and PLTs determined by blood counter (HEMAVET) 2 h after CASIN administration (IV, 0.5 mM, 200 μl/20 g mouse weight) in comparison to controls. Middle and right: Numbers of myeloid-, erythroid-, T-, and B- lineage cells determined by flow cytometry (CD11b, Ter119, CD3, and B220) 2 h after CASIN administration (1.2 mg/kg, IV) in comparison to controls in both BM and PB. Results are representative of at least three independent experiments

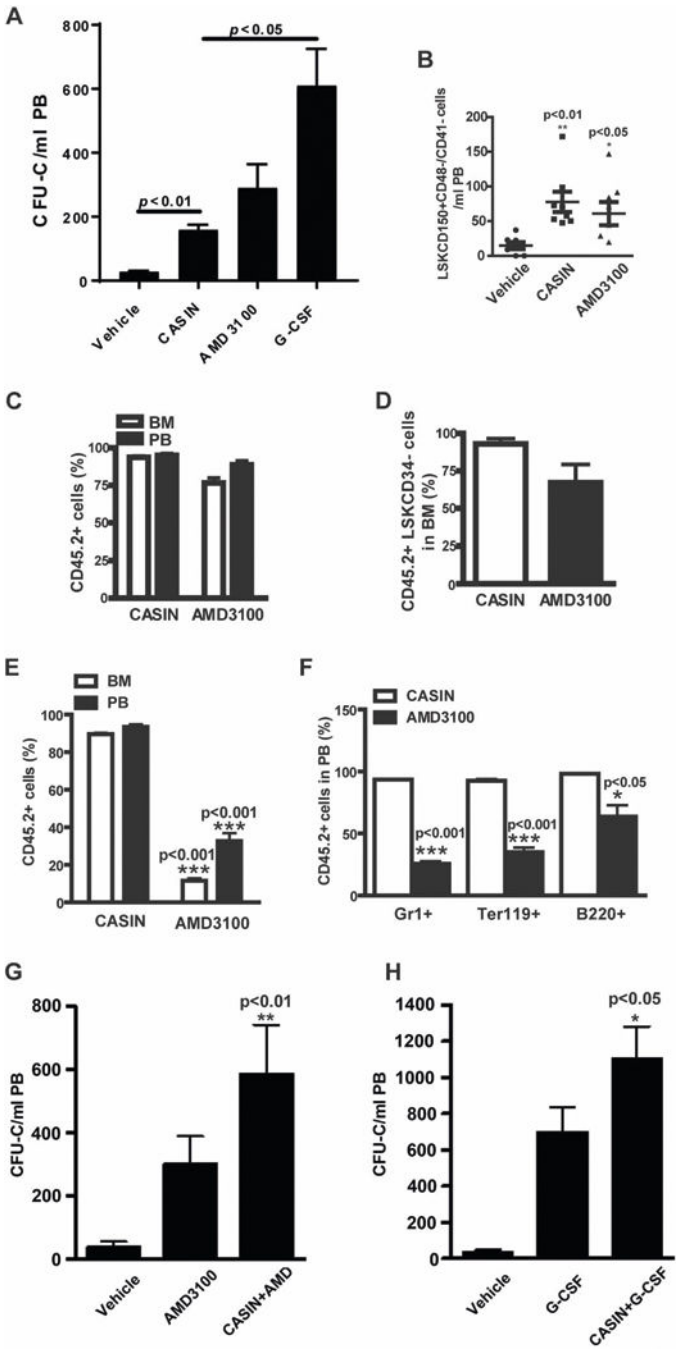

Fig. 6.

CASIN-mobilized HSCs are superior in functionality to those mobilized by AMD3100. **a** Mobilization of hematopoietic progenitor CFU activities in peripheral blood post-CASIN, AMD3100 or G-CSF injection. PB from the mice treated with CASIN (1.2 mg/kg, IV), AMD3100 (5 mg/kg, IP), or G-CSF injection (100 μg/kg per day once a day for 5 days, IP) were subjected to CFU assay. ($n = 5$ per group). Results are representative of at least three independent experiments. **b** Quantification of LSKCD150^{+}CD48^{-}CD41^{-} cells in PB of mice mobilized with CASIN, AMD3100, or vehicle 2 h post injection of CASIN (1.2 mg/kg, IV)

(*n* = 8), AMD3100 (5 mg/kg, IP) (*n* = 8), or vehicle (control, *n* = 7). **c** The percentages of CD45.2 donor leukocytes in CD45.1-recipient mice in both PB and BM of lethally irradiated primary recipients transplanted with PBMCs (500 μl blood) from mice subjected to CASIN or AMD3100 injection. Results are representative of at least three independent experiments. **d** The percentages of CD45.2 donor LSKCD34$^-$ HSCs in CD45.1-recipient mice in the BM of lethally irradiated primary recipients transplanted with PBMCs (500 μl blood) from mice subjected to CASIN or AMD3100 injection. Results are representative of at least three independent experiments. **e** CASIN and AMD3100 mobilized long-term HSC activities were assayed by secondary transplantation into lethally irradiated BoyJ mice (5×10^6 donor BM cells from primary recipients for each secondary recipient; *n* = 7 and 6 for CASIN and AMD3100 groups, respectively). The percentages of CD45.2$^+$ donor cells in both PB and BM of secondary CD45.1-recipients were analyzed by FACS 6 months after the secondary transplant. Results are representative of at least three independent experiments. **f** The Gr1$^+$, Ter119$^+$, and B220$^+$ lineages of CD45.2 donor cells in PB of secondary CD45.1-recipients were analyzed 6 months after the secondary transplant. Results are representative of at least three independent experiments. **g** Mobilization of progenitor CFU activity by AMD3100 alone or in combination with CASIN. AMD3100 (5 mg/kg, IP) (*n* = 6) or AMD3100 (5 mg/kg, IP) in conjunction with CASIN (1.2 mg/kg, IV) (*n* = 6) treated mice were analyzed for CFU-C activities in PB 2 h after the respective treatment. Results are representative of at least three independent experiments. **h** Mobilization of progenitor CFU activity by G-CSF alone or in combination with CASIN. G-CSF (100 μg/kg per day once a day for 5 days, IP) (*n* = 4) or G-CSF in conjunction with CASIN (1.2 mg/kg, once in day 6 of G-CSF, IV) (*n* = 5) treated mice were analyzed for CFU-C activities in PB 2 h after the CASIN treatment. Results are representative of at least three independent experiments