Diaphanous-related formin mDia2 regulates beta2 integrins to control hematopoietic stem and progenitor cell engraftment

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Bone marrow engraftment of the hematopoietic stem and progenitor cells (HSPCs) involves homing to the vasculatures and lodgment to their niches. How HSPCs transmigrate from the vasculature to the niches is unclear. Here, we show that loss of diaphanous-related formin mDia2 leads to impaired engraftment of long-term hematopoietic stem cells and loss of competitive HSPC repopulation. These defects are likely due to the compromised transendothelial migration of HSPCs since their homing to the bone marrow vasculatures remained intact. Mechanistically, loss of mDia2 disrupts HSPC polarization and induced cytoplasmic accumulation of MAL, which deregulates the activity of serum response factor (SRF). We further reveal that beta2 integrins are transcriptional targets of SRF. Knockout of beta2 integrins in HSPCs phenocopies mDia2 deficient mice. Overexpression of SRF or beta2 integrins rescues HSPC engraftment defects associated with mDia2 deficiency. Our findings show that mDia2-SRF-beta2 integrin signaling is critical for HSPC lodgment to the niches.
Hematopoietic stem and progenitor cells (HSPCs) reside in bone marrow in adults where stromal cells provide a favorable microenvironment for their proliferation and differentiation. Under certain circumstances, cytokine-stimulated HSPCs migrate through the bone marrow–blood barrier and enter the peripheral blood. The ability of HSPCs to migrate and engraft allow bone marrow transplantation (BMT) to be used as a routine clinical strategy to treat patients with various hematologic diseases. After transplantation, the donor HSPCs rapidly localize to the central marrow region, where they redistribute to produce preferential seeding of long-term hematopoietic stem cells (LT-HSCs) that are close to the endosteal region. After this homing process, long-term engraftment of HSPCs eventually leads to repopulation of various hematopoietic lineages. Although previous studies revealed critical factors involved in HSPC homing and engraftment, how these cells transmigrate from the vasculatures to the niches is unclear.

Regulation of the membrane cytoskeleton network by actin polymerization has been demonstrated to be critical for HSPC homing, migration, and engraftment. In hematopoietic cells, there are two major actin-nucleating protein families: the Wiskott–Aldrich syndrome protein (WASP)-Arp2/3 protein complexes and diaphanous-related formin proteins that nucleate branched and unbranched F-actin filaments, respectively. The diaphanous-related formins, including mDia1, 2, and 3, are characterized by a unique and highly conserved actin polymerization formin homology 2 (FH2) domain that is preceded by a proline-rich FH1 domain. These two enzymatic domains are flanked by the N-terminal Rho GTPase-binding domain (GBD) and the C-terminal diaphanous auto-regulatory domain (DAD). The FH1 domain, in a complex with profilin, recruits G-actin subunits to the FH2 domain for polymerization of linear actin filaments. The diaphanous-inhibitory-domain (DID) and the dimerization domain (DD) are required for subcellular localization of mDia formins. Biochemical studies showed that the DID and DAD domains interact to form an auto-inhibitory structure. This auto-inhibition of mDia formins is further supported by findings showing that mDia formin variants with mutations in the DID or N-terminal regions are constitutively active. When activated Rho GTPase binds to the GBD domain, the auto-inhibitory loop is relieved and mDia formins are activated. mDia formins can also be activated through post-translational modifications.

In many cell types, mDia formins are involved in diverse processes including cytokinesis, cell polarity, endocytosis, filopodium formation, adhesion, and migration. The dynamics of cell adhesion and motility are tightly regulated by actin cytoskeleton rearrangements. This regulation is particularly important for the activity of various hematopoietic cells, such as immune cell migration in response to antigens and HSPC engraftment. Although WASP is known to be required for HSPC migration and engraftment, whether mDia formins are involved in these processes is unclear. Several studies have shown that mDia formins play important functions in hematopoietic cells including T cells, neutrophils, macrophages, and erythroid cells. Many of these studies used knockout mouse models to reveal the functions of mDia in vivo. mDia1-deficient mice are viable, but develop symptoms that mimic age-associated human myelodysplastic syndromes. Mice with global knockout of mDia2 are embryonically lethal with defects in fetal erythropoiesis. We recently generated a mDia2 conditional knockout mouse model. Mice with hematopoietic-specific knockout of mDia2 exhibit anemia with many bi-nucleated late-stage erythroblasts and significant defects in terminal erythropoiesis in the bone marrow. In this study, we use these mouse models to reveal that mDia2 plays a critical role in HSPC engraftment. In particular, we find that loss of mDia2 does not compromise HSPC homing to the bone marrow vasculatures. Instead, mDia2 is essential for transendothelial migration from the vasculature and subsequent lodging in bone marrow niches. We further reveal the presence of a mDia2–MAL–SRF–beta2 integrin signaling axis that mediates mDia2 function in HSPC bone marrow lodging.

**Results**

**Loss of mDia2 does not affect steady-state HSPC compositions.**

The mDia formin proteins in mice include mDia1, mDia2, and mDia3 that are encoded by Dia1, 3, and 2, respectively. Prior studies demonstrated that mDia2 is enriched in the hematopoietic cells. Using a quantitative real-time PCR, we confirmed that mDia2 is highly expressed in HSPCs (Supplementary Fig. 1a). By analysis of the Gene Expression Commons database, we also found that mDia2 is enriched in HSPC populations (Supplementary Fig. 1b).

To determine the role of mDia2 in HSPC function, we first analyzed the content of HSPCs in a mDia2fl/flVav-Cre mouse model in which mDia2 deletion specifically in hematopoietic tissue initiated on embryonic day 12. The HSPCs we analyzed include LSKs (lineage negative, Sca-1−, c-Kit−), Lks (lineage negative, c-Kit+, Sca1−), multipotent progenitors (MPPs, LSK, CD34+, CD135+), long-term HSCs (LT-HSCs LSK, CD34−, CD135−), short-term HSCs (ST-HSCs, LSK, CD34+, CD135−), and SLAM (CD150+, CD48−, LSK) HSCs as we performed previously. These mice showed anemia as reported. However, there were no significant changes on HSPC contents in the bone marrow (Supplementary Fig. 1c, d). In addition, cell cycle analysis showed minimal changes in HSPC quiescence in mDia2-deficient mice (Supplementary Fig. 1e, f). Together, these results indicate that mDia2 may not be required to maintain the steady state of HSPCs, which prompted us to focus on the role of mDia2 in HSPC homing and engraftment during BMT.

**mDia2 is required for HSPC engraftment in transplantation.**

BMT represents a stress condition for HSPCs. We first performed a transplantation assay in which CD45.2+ bone marrow mononuclear cells (BMMC) from control or mDia2fl/fl Vav-Cre mice were transplanted non-competitively into lethally irradiated CD45.1+ recipient mice. Compared to mice transplanted with wild-type (WT) control bone marrow cells, mice transplanted with mDia2 null cells were anemic, which is consistent with our previous report. When we analyzed the HSPCs 4 months after transplantation, LT-HSC and ST-HSC populations were proportionally increased (Fig. 1a, b) and a trend toward increases in the absolute cell number of the HSPCs in the bone marrow was observed (Fig. 1c).

Analysis of the cell cycle status of the LSK population in recipient mice transplanted with BMMCs from mDia2fl/fl Vav-Cre mice showed cells increased in G1 but decreased in G0 phases (Fig. 1d, e). This result suggests a loss of quiescence in mDia2-deficient LSK cells after BMT, which could lead to the initial expansion but later exhaustion of HSPCs. Indeed, when we analyzed these mice 10 months after transplantation, we observed significantly reduced LSK and LS cells (Supplementary Fig. 2a) and a continued loss of quiescence (Supplementary Fig. 2b). These phenotypes were also observed in the plpC-treated mDia2fl/flMx-Cre mouse model, an inducible system that enables hematopoietic-specific mDia2 knockout in adult animals (Supplementary Fig. 2c, d). Mice transplanted with mDia2-deficient bone marrow cells also exhibited increased lethality (Fig. 1f). When we transplanted mice with bone marrow cells from primary transplantation recipients, mice transplanted with mDia2-deficient bone marrow had markedly shortened survival
Fig. 1 mDia2 is required for HSPC engraftment in bone marrow transplantation. a Representative flow cytometric plots showing gating of different HSPC subpopulations in bone marrow lineage negative cells from wild type mice 4 months after transplantation with $2 \times 10^6$ BMMCs from control or mDia2fl/fl Vav-Cre mice. b The percentages of indicated HSPC subpopulations in a. c The absolute number of indicated HSPCs in a. d Representative flow cytometric plots of cell cycle profile of LSK cells in a. e Quantitative analyses of d. n = 5 mice in mDia2fl/fl, n = 6 in mDia2fl/fl Vav-Cre for both b, c, and e. f Kaplan-Meier survival curve of mice transplanted with $2 \times 10^6$ BMMCs from control or mDia2fl/fl Vav-Cre mice. n = 17 mice in each group. To further investigate the role of mDia2 under BMT stress conditions, we performed a competitive BMT (cBMT) assay in which an equal number of BMMCs from CD45.2+ mDia2fl/fl Vav-Cre mice and CD45.1+ congenic WT mice were transplanted into lethally irradiated wild type CD45.1+ recipient mice (Fig. 2a). Testing of peripheral blood chimerism 5 weeks after transplantation revealed that the absence of mDia2 elicited an...
almost complete loss of CD45.2+ cells in the peripheral blood compared to the WT littermate controls (Fig. 2b). Moreover, lineage analyses confirmed a near absence of neutrophils, B, and T cells derived from mDia2-deficient donors (Fig. 2c). Loss of competitive reconstitution of the mDia2-deficient BMSC was also found in the bone marrow and spleen (Fig. 2b, c). Importantly, the absence of mDia2-deficient cells in the LSK, LK, LT-HSC, ST-HSC, and multipotent progenitor (MPP) populations (Fig. 2b, c) demonstrated that this competitive reconstitution defect was not due to blockage of HSPC differentiation. Engraftment defects were evident from 1 to 12 months after transplantation, indicating that both short-term progenitor and long-term stem cell engraftment were affected by the loss of mDia2 (Fig. 2d). These competitive engraftment...
defects of mDia2-deficient HSPCs were also observed in the plpC-treated mDia2fl/flMx-Cre mouse model (Supplementary Fig. 2e–g). We further confirmed that this defect in engraftment was due to cell-intrinsic loss of mDia2 in HSPCs, since mDia2fl/flVav-Cre BMCCs from the primary transplants were also significantly reduced in subsequent competitive transplantations (Supplementary Fig. 2h, i).

mDia2 is required for HSPC lodgment and mobilization. Defects in the competitive transplantation assay involving mDia2 knockout bone marrow could also be due to compromised short-term HSPC homing. To test this possibility, we first analyzed the expression levels of CXCR4, which senses SDF-1 and is critical for HSPC homing and engraftment, in mDia2-deficient HSPCs. In comparing control and mDia2-deficient HSPCs, we observed no significant changes in CXCR4, cell death or ex vivo proliferation profiles (Supplementary Fig. 3a–c). We next performed an in vivo homing assay and found that mDia2-deficient HSPCs migrated to the bone marrow 12, 24, and 48 h after BMT to a degree that was comparable to the WT controls (Fig. 2e, Supplementary Fig. 3d–f). These data indicate that the initial HSPC bone marrow localization is not affected by the loss of mDia2 in vivo. However, this short-term homing assay cannot detect defects in trans-endothelial migration, a key process for HSPC lodgment. Indeed, loss of mDia2 was associated with a reduction in HSPC trans-well migration capacity in vitro in response to SDF1α in the presence or absence of endothelial cells (EC) (Fig. 2f, g). To directly determine trans-endothelial migration in vivo, we performed an imaging assay to visualize fluorescently labeled donor HSPCs and sinusoidal vessels in whole-mount long bones from the recipient animals 18 h after competitive transplantation. We found that many donor HSPCs from mDia2fl/flVav-Cre mice were either trapped inside the vasculature or associated with the vessels, whereas the WT counterparts were readily detected outside of the vessels in the presence of CD45.1+ congenic competitors (Fig. 2h, i, Supplementary Fig. 4a). To confirm this finding under more physiologically relevant conditions, we performed a transplantation assay in non-irradiated recipient mice. The recipient mice exhibited the same trans-endothelial migration defects in mDia2-deficient HSPCs in the bone marrow and spleen (Supplementary Fig. 4b, c). We also performed a competitive transplantation assay involving injection of donor BMMCs directly into the bone marrow through an intrafemoral route, which partially circumvented the engraftment defect in mDia2-deficient HSPCs (Supplementary Fig. 4d).

Lack of trans-endothelial migration could also lead to the reduction in G-CSF-induced HSPC mobilization out of the niche. To test this, we treated mDia2fl/flVav-Cre mice with G-CSF to induce HSPC mobilization. We found that mDia2fl/flVav-Cre mice had significantly fewer HSPCs in circulation compared to control mice (Fig. 2j, k). Taken together, these results reveal that mDia2 is required for both HSPC engraftment and lodging in the bone marrow niche.

mDia2-SRF signaling is involved in HSPC engraftment. mDia formin proteins are critical for linear actin polymerization. We indeed observed significant downregulation in F-actin filaments in c-Kit+ HSPCs from mDia2-deficient mice (Fig. 3a–c). Downregulation of F-actin was also associated with the changes in cell polarity in which the bipolar distribution of F-actin and alpha-tubulin that is commonly observed in the wild type HSPCs was absent in mDia2-deficient cells (Fig. 3a, b). In addition, the prevalence of F-actin protrusions (Fig. 3a arrows) was significantly diminished in mDia2-deficient HSPCs (Fig. 3d).

Dysregulation of the actin cytoskeleton associated with loss of mDia2 was reported to affect cell functions through inactivation of the MAL-SRF pathway in cell-based assays. In this respect, mDia formin-mediated actin polymerization consumes G-actin monomers from MAL (also known as MRTFa and MKL1). The released MAL rapidly accumulates in the nucleus where it dimerizes with serum response factor (SRF) and drives SRF-dependent gene expression. Supporting this hypothesis, recent genetic studies demonstrated that both SRF and MAL are required for HSPC colonization of the bone marrow and chemotaxis in response to SDF1α. To determine whether the actin–MAL–SRF axis is impaired by loss of mDia2 in vivo, we stimulated the c-Kit+ HSPCs with serum, which induced MAL nuclear localization in the wide-type cells. However, in HSPCs from mDia2fl/flVav-Cre mice, MAL maintained a cytoplasmic localization (Fig. 3e and Supplementary Fig. 5a), indicating that the SRF transcriptional network could be disrupted in mDia2-deficient HSPCs. Therefore, we performed a quantitative RT-PCR analysis of c-Kit+ HSPCs purified from mDia2fl/flVav-Cre mice. We found that many established SRF target genes, including Acta2, KRT17, Flna, FHLL2, Myh9, Actb, and Actg1, were significantly down-regulated relative to the control (Fig. 3f).

The level of SRF itself was slightly decreased by the loss of mDia2, consistent with the fact that SRF is also a reciprocal transcriptional target of itself. SRF can influence the expression of various adhesion molecules. Given the role of mDia2 in HSPC trans-endothelial migration, we next examined the expression of several integrins that are critical for HSPC adhesion to EC and trans-endothelial migration. Indeed, the mRNA levels of beta2 subfamily integrins,
including ItgaL, ItgaM, and Itgb2 that encode CD11a, CD11b, and CD18, respectively, and are known to mediate EC interactions, were significantly decreased in bone marrow c-Kit+ HSPCs from mDia2fl/fl Vav-Cre mice (Fig. 3g). Serum stimulation dramatically induced the expression of ItgaM, Itgb2, and the classic SRF downstream target genes. The decreased expression of these genes was more prominent in mDia2 null cells (Supplementary Fig. 5b, c). The surface expression of these integrins was also consistently decreased in HSPCs from mDia2fl/fl Vav-Cre mice and ex vivo cultured mDia2 null HSPCs (Fig. 3h–j). The expression of beta2 integrins in HSCs was demonstrated in a gene expression database in that the reading peaks in RNA-sequencing and H3K36 trimethylation (an epigenetic mark associated with the activation of RNA polymerase 2) ChIP-sequencing were evident.
for both ItgaM and Itgb2. In contrast, transcription of proximal Itgad loci near ItgaM was barely detectable (Supplementary Fig. 5d–g). To determine whether downregulation of these integrins affects HSPC adhesion, we performed an adhesion assay using ex vivo cultured HSPCs and revealed compromised adhesion of mDia2-deficient HSPCs to several bone marrow extracellular matrix components (Fig. 3k) that have been shown to bind to beta2 integrins in hematopoietic cells44,45. In coverslips coated with ICAM-1, a main physiological substrate of beta2 integrins46, mDia2-deficient HSPCs also showed adhesion defects (Fig. 3l). Collectively, these data indicate that EC binding and trans-endothelial migration defects in mDia2-deficient HSPCs are likely due to altered expressions of beta2 integrins mediated by the MAL–SRF pathway.

**Beta2 subfamily integrins are targets of SRF.** Homodimeric SRF binds to a consensus cis-element, known as the CARG box (CC(A/T)6GG) or serum response element (SRE), on its target genes47,48. To determine whether beta2 integrins are bone fide transcriptional targets of SRF, we used a SRF chromatin-binding database49 with a transcription factor-binding site prediction tool (TFBIND)50 and identified intrinsic binding of SRF on both ItgaM and Itgb2 genomic loci (Fig. 4a), but not on Itgad. Therefore, we focused subsequent studies on ItgaM and Itgb2. SRF presents as several isoforms with exon 3, 4, or 5 deleted singly or in combination (Fig. 4b). In addition to the full-length SRF (SRF-FL), the isoform with deletion of exon 5 (SRFΔ5) was also highly expressed in sorted NK and LSK cells (Fig. 4b). We cloned the intronic region of either ItgaM or Itgb2 that contains the predicted SRE and inserted it ahead of the luciferase gene (Fig. 4c). As expected, full-length SRF strongly and dose-dependently induced luciferase gene expression when co-transfected with luciferase reporters containing either the ItgaM or Itgb2 intronic region in 293T cells (Fig. 4d, e). Notably, the orientation of these SRE elements exhibited marked differences in the ability to enhance luciferase activities, indicating a possible complex three-dimensional chromatin organization in vivo (Fig. 4c). SRFΔ5-activated luciferase activity to a lesser degree compared to SRF-FL, but the level was comparable to the constitutively active SRF-VP16 in 293T cells (Supplementary Fig. 6a). To further validate these SREs, we mutated the consensus C/G nucleotides (AA mutant), the adjacent T to C (TC mutant), or these two sites combined (AAGT mutant) in the intronic SRE of ItgaM. Each change was associated with dramatic reductions in SRF-induced luciferase activities (Fig. 4f). The SRE on Itgb2 intron is non-classic. Deletion of this site blocked the luciferase activity (Fig. 4g). Since C/G is critical for the consensus sequence, we mutated C and G in this non-classic SRE site to produce a G/C to T mutation. As expected, this mutation also abolished luciferase activity (Fig. 4g). Using chromatin immunoprecipitation (ChiP) assays of c-kit+ HSPCs, we further confirmed SRF and MAL binding to these SREs, as well as SRFs established target Acta2, which was abolished by the loss of mDia2 (Fig. 4h and Supplementary Fig. 6b). Next, we depleted the intrinsic SRE of ItgaM in HSPCs using a CRISPR-Cas9 system, which also significantly affected CD11b expression (Fig. 4i and Supplementary Fig. 6d–g). Overall, these results reveal functional SRF cis-elements in beta2 integrins.

**SRF restores engraftment defects in mDia2-deficient HSPCs.** To establish a proof-of-concept, we next analyzed whether SRF overexpression could rescue the engraftment defects in vivo in mDia2-deficient HSPCs. We first showed that overexpression of SRF-FL or SRFΔ5 rescued protein expression of beta2 integrins in mDia2fl/fl Vav-Cre HSPCs in vitro (Fig. 5a and Supplementary Fig. 7a). A constitutively active mDia2 mutant with a deletion of the C-terminal DAD domain (ΔDAD) also strongly rescued beta2 integrin expression even with a low transduction efficacy that was partially due to the insertion of a large open-reading frame (ORF) into the vector. In contrast, an N-terminal GBD/DID mutant lacking actin polymerization domains had no rescue effect, further emphasizing the significance of actin polymerization activity in mDia2-mediated expression of beta2 integrins. We also examined multiple mRNA transcripts in mDia2-deficient HSPCs after SRF overexpression by quantitative RT-PCR. Both SRF-FL and Δ5 isoforms normalized the mRNA levels of beta2 integrins and several known SRF target genes to wild type levels (Fig. 5b and Supplementary Fig. 7e). In addition, SRF overexpression rescued the compromised adhesion ability of mDia2-deficient HSPCs in vitro (Fig. 5c).

To determine the rescue effects of SRF in vivo, we used competitive transplantation assays in which SRF-FL, SRFΔ5, or
mDia2 mutants were transfected into CD45.2+ c-Kit+ HSPCs cells from mDia2fl/fl Vav-Cre mice. Equal numbers of these cells were transplanted with an equal number of empty vector-transduced wild type CD45.1+ HSPCs into lethally irradiated CD45.1+ recipient mice. Chimeras in peripheral blood could be observed by flow cytometry for up to 6 months post-transplantation (Fig. 5d). Re-expression of WT mDia2 or the constitutively active mDia2 mutant (ΔDAD) completely rescued the engraftment defects in competitive transplantation of mDia2-deficient HSPCs in vivo, even with low transduction efficiencies (Fig. 5e and Supplementary Fig. 7f). Overexpression of both SRF-FL and SRFΔ5 also significantly rescued the
competitive engraftment defects in mDia2-deficient HSPCs (Fig. 5e). The rescue effects persisted over the long term and in multi-lineages (Fig. 5e, f). More importantly, CD45.2+ LT-HSC and LSK cells were readily detected at 6 months post-transplantation in these rescued mice (Fig. 5g). To further demonstrate the significance of SRF in mediating the functions of mDia2 in HSPC engraftment, we performed a serial transplantation assay in which various mDia2 or SRF constructs were transduced into mDia2-deficient c-Kit+ HSPCs before use in serial transplants. As expected, mDia2 WT, ΔΔD, and SRF-FL, SRF-Δ5 significantly extended the survival of the recipients with a secondary bone marrow transplant (Fig. 5h). Taken together, these results demonstrate that mDia2 regulates HSPCs in vivo mainly through the SRF signaling.

**Beta2 integrins mediate mDia2 function in HSPC engraftment.** CD18 interacts with CD11a and CD11b to form LFA1 and Mac1, respectively, which are critical for hematopoietic cell migration and adhesion. However, the roles of these complexes in HSPC engraftment are unclear. To study hematopoietic-specific loss of function of beta2 integrins in vivo, we constructed hematopoietic-specific knockout animal models of CD11b or CD18 deficiency using CRISPR-Cas9 (Fig. 6a). Before the sgRNAs were introduced into HSPCs, their targeting efficacy was measured through a rapid and quantitative luciferase activity-based assay. Specifically, the sgRNA target sites on CD11b or CD18 were cloned in-frame within the region of the luc2P gene encoding the N-terminus. The clones were then co-transfected with sgRNA expression vectors into 293T cells stably expressing CRISPR-associated protein 9 (Cas9). The effective Cas9:sgRNA complex is expected to bind to target DNA sequences causing out-of-frame indels that abrogate the luciferase activity (Fig. 6a). Using this approach, we obtained two sgRNAs that separately targeted ItgaM and Itgb2 with over 90% efficiency (Fig. 6b). We next transduced sgRNA lentiviral particles into c-Kit+ HSPCs from mice that constitutively and ubiquitously express Cas9 and EGFP and transplanted these cells into lethally irradiated CD45.1+ recipient mice (Fig. 6a). We archived nearly 90% transduction efficiency as verified in peripheral blood mononuclear cells from these mice (Fig. 6c). As a consequence, cell surface expression of CD18 and CD11b was almost completely abolished in Gr1+ granuloocytes and LSK cells (Fig. 6d, e). Thus, we obtained mice with either separate ItgaM or Itgb2 deficiency or deficiency of both genes in hematopoietic cells. In competitive transplantation assays, knockout of CD11b or CD18 individually or together was associated with failure of competitive engraftment (Fig. 6f). We also performed secondary BMT assays to test the engraftment and self-renewal capacity of HSCs after loss of beta2 integrins. Recipient mice transplanted with HSPCs from combined CD11b and CD18 knockout died rapidly after transplantation compared to mice transplanted with HSPCs from mice lacking only CD18 or control HSPCs (Fig. 6g). Collectively, our data suggest that beta2 integrins are indispensable for HSPC engraftment and long-term self-renewal of HSCs.

We next tested whether mDia2 deficiency-induced defects in HSPC engraftment could be rescued by ectopic expression of beta2 integrins. We found that ectopic expression of ItgaM, but not Itgb2, partially restored the competitive engraftment defect in mDia2ΔΔD Vav-Cre c-kit+ HSPCs (Fig. 6h, i). Co-ectopic expression of ItgaM and Itgb2 reverted the rescue effect of ItgaM (Fig. 6i). These data reveal that ItgaM, but not Itgb2, is the limiting factor on the mDia2-SRF signaling cascade involved in HSPC engraftment. They also indicate that CD18 levels must be precisely regulated to avoid adverse effects on HSPCs mediated by CD18 overexpression.

**Discussion** Successful bone marrow engraftment requires HSPC homing to the bone marrow vasculature, trans-endothelial migration, and lodging to the bone marrow niche. Research over the past few decades has revealed proteins that play key roles in HSPC homing and engraftment including selectins, ICAM-1, VCAM-1 on the ECαvβ3, β1 integrin subfamilies, and CXCR4 on HSPCs. However, the mechanisms involved in trans-endothelial migration of HSPCs were unclear since loss of function studies focusing on these proteins showed HSPC defects mainly in short-term homing or long-term engraftment processes. Although vascular endothelial cadherin was indicated to play a role in HSPC migration across bone marrow endothelium, proteins expressed on HSPCs in the trans-endothelial process are rarely studied. In this study, we reveal a role for the formin protein mDia2 in the engraftment of HSPCs during transplantation. The mDia2 hematopoietic-specific knockout mouse model overcomes the embryonic lethality of whole body mDia2 knockout and provided us an opportunity to determine the role of mDia2 in HSPCs in adults. We found that loss of mDia2 in adult mice significantly compromised competitive HSPC engraftment and long-term HSC repopulation capacities. Surprisingly, HSPC localization to the bone marrow vasculature in these mice remained intact. Through imaging analyses, we revealed that mDia2 is critical for the trans-endothelial migration of HSPCs. mDia2 is also critical for HSPC adhesion to the bone marrow niche. Here we found decreased adhesion of mDia2 knockout HSPCs to extracellular matrix proteins in vitro. Loss of mDia2 was associated with a slight expansion of HSPCs in the bone marrow niche.
Fibronectin

HSC quiescence defects continued through the late stages of transplantation that eventually led to exhaustion and reduction in HSPC populations.

We further demonstrated that mDia2 functions through the MAL–SRF signaling axis to regulate HSPC engraftment. Overexpression of SRF, including a naturally occurring SRF isoform with a deletion of exon 5, rescued the engraftment and long-term HSC reconstitution defects in mDia2-deficient HSPCs. These results are consistent with a previous study on the role of SRF in

marrow along with loss of HSC quiescence in the early stages after transplantation. This result is consistent with previous studies that ICAM-1 deficiency in the bone marrow niche impairs HSC quiescence. The defects in HSC quiescence defects continued through the late stages of transplantation that eventually led to exhaustion and reduction in HSPC populations.
Fig. 5 SRF restores engraftment defects in mDia2-deficient HSPCs. a Quantitative analysis of the flow cytometry detected surface expression levels of beta2 integrins in c-Kit+ HSPCs from mDia2fl/fl or mDia2fl/flVav-Cre (cKO) mice transduced with indicated genes. N= 3 mice in each group, except mDia2 ΔDAD and mDia2 GBD/DID groups. b Restoration of the mRNA expression of beta2 integrins and indicated SRF downstream genes by overexpression of SRF in c-Kit+ cells from mDia2fl/flVav-Cre (cKO) mice. The experiments were performed in triplicate. N = 3 mice in each group. c Viral transduced c-Kit+ HSPC from mDia2fl/fl or mDia2fl/flVav-Cre (cKO) mice were applied for in vitro adhesion assay with fibronectin precoated coverslip for 1 h at 37 °C. Adherent cells were randomly scored from N = 7 fields in mDia2fl/fl+Vector, N = 9 fields in cKO+Vector, N = 8 fields in cKO+SRFΔ55, and N = 6 fields in cKO+SRF-FL. Results are expressed as the adhesion ability relative to control cells transduced with vector. d Schematic diagram showing bone marrow transplantation experiments in e-g. e Chimera studies of peripheral blood mononuclear cells derived from c-Kit+ cells that were transduced with the indicated constructs after competitive transplantation with CD45.1 with the E2A transcription factor to facilitate their expression61. f Mean–median and box plots of SRF-FL expression in indicated constructs after secondary transplantation illustrated in d. Error bars represent the SEM of the mean. p values compared to cKO-Vec group from j. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Two-tailed unpaired Student’s t test was used to generate the p values. Significance for survival analyses was calculated by log-rank (Mantel–Cox) test.

HSPC engraftment and adhesion to bone marrow niches40. In contrast to the mouse model with hematopoietic-specific loss of SRF that showed a significant expansion in HSPCs, we observed no changes in these populations in mDia2-deficient mice under steady-state conditions. Instead, the HSPC defects were mostly observed during BMT. This feature could be due to a compensatory effect of mDia1. Furthermore, given that SRF regulates multiple target genes, loss of SRF could induce HSPC phenotypes under steady-state conditions that were caused by other genes targeted by SRF.

In this respect, the role of mDia2 in HSPC function is apparently more specific than that of SRF in that the mDia2–MAL–SRF axis is critical for trans-endothelial migration that is part of the complex journey taken by HSPCs during homing and engraftment. This specificity is also evident in that only a subset of SRF downstream targets is affected by the loss of mDia2 in HSPCs. Among these down-regulated genes, we discovered that beta2 subfamily integrins, including ItgαM and Itgβ2, are direct SRF target genes. Beta2 integrins are mainly expressed in granulocytic cells. Accumulating evidence demonstrates that these integrins are also expressed on HSPCs. For example, a large proportion of the side population of HSCs co-express low levels of CD11559 and nearly half of phenotypic mouse HSCs (defined as CD150+CD34−LSK) are CD11a positive60. Furthermore, long non-coding RNAs specifically expressed in HSCs (LncHSCs) co-occupy Itgβ2 promoter regions with the E2A transcription factor to facilitate their expression61. These integrins are also functionally important for HSPCs. Specifically, Mac1 (CD11b/CD18 complex) mediates the adhesion of hematopoietic progenitor cells to stromal cell elements52. Meanwhile, ITGAM was reported to colocalize with GPI-80, which is critical for human HSC in vitro expansion and engraftment63.

We further demonstrated the significance of beta2 integrins in regulating the mDia2–SRF pathway by showing that ItgαM overexpression can rescue defects in competitive transplantation of mDia2 null HSPCs. However, Itgβ2 overexpression failed to rescue this phenotype. We thus hypothesize that CD18 levels must be precisely regulated in that retroviral-mediated overexpression could be toxic to HSPCs. Consistent with this hypothesis, overexpression of both ItgαM and Itgβ2 also failed to rescue the defect. The complexity of CD18 activity was highlighted by earlier in vivo studies involving a hypomorphic CD18 mutant mouse model that revealed an expansion of HSPCs in the bone marrow in competitive transplantation assays64. In the present study we found that CD18-deficient HSPCs, generated through a CRISPR-Cas9 approach, lost their competitive engraftment capacities in a manner similar to that seen for mDia2 deficiency. This discrepancy indicates that hypomorphic CD18 could have potential gain-of-function properties.

SRF recognizes a SRE on its target genes that is commonly known as the CARG element (CC(A/T)6GG)48. SREs are typically classified into two categories, consensus CARG and CARG-like48. The SRE on ItgαM is consistent with the CARG-like element. Although the SRE on Itgβ2 is non-classical, recent studies using SRF ChIP coupled with next generation sequencing (ChIP-seq) revealed SREs that lack CC(A/T)6GG consensus sequence in many SRF-regulated genes65. These SREs on ItgαM and Itgβ2 are also orientation-dependent as demonstrated by the luciferase assay in this study that suggested a complex chromosome organization for SRF binding and regulation via these elements. This was further indicated by an incidental finding that a thymidine nucleoside 3′ downstream of the ItgαM’s SRE is critical for the recognition of SRF. However, it is also possible that this site affects binding of another factor that could be required for SRF to be functional.

We previously reported that loss of mDia1 compromised endocytosis and led to dysregulation of CD11b on neutrophil cell membranes22. In contrast, findings in the current study demonstrated that expression of beta2 subfamily integrins, including CD11b, is downregulated both at the mRNA and cell membrane protein level in the HSPCs from mDia2-deficient mice. Notably, these cell type-specific functions are also found in SRF66. In this respect, previous studies in neutrophils revealed that loss of SRF did not significantly affect the mRNA level of many integrins. Instead, cell membrane expression levels of beta2 integrins were upregulated in SRF null neutrophils due to the lack of internalization and recycling of these integrins67. The findings of the present study are consistent with the finding that upregulated CD11b in mDia1 null neutrophils is due to compromised endocytosis activity and suggests a possible connection of mDia1 with SRF in neutrophils as well. Moreover, these studies indicate that, similar to SRF, mDia forms also play cell type-specific and content-specific roles in that in HSPCs, mDia mainly influences the transcriptional activity of SRF to affect beta2 integrin expression, whereas in neutrophils, mDia mainly functions to regulate integrin endocytosis to affect their cell membrane expression.

Methods

Mice. Genetically modified tissue-specific mDia2 knockout mice with a C57/BL6 background were described previously25,26. Congenic mice carrying CD45.1 antigen were purchased from Charles River (B6-LY-5.2/Cr, strain code: 564). C57/BL6 WT mice, CAG-Cas9 transgenic mice (stock #026179), Mx-Cre mice (stock #00356), and Vav-Cre mice (stock #008610) were purchased from the Jackson Laboratory. All the experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Northwestern University.

Expression constructs. The MSCV-IRES-hCD4 (MIDC4) construct was described previously. All inserts were sub-cloned 5′ to the IRES32. Murine SRF ORF was PCR amplified using cDNA reverse transcribed from total RNA of mouse bone marrow lineage negative cells. Two isoforms including SRF full length (SRF-FL) and exon 5-deficient truncation (SRFΔ55) were obtained. The expression construct was microinjected into pronuclear-stage mouse eggs to generate transgenic mice.
carrying a HA-tagged, constitutively active form of human SRF was kindly provided by Dr. Naren Ramanan (Centre for Neuroscience Indian Institute of Science, India), and was PCR amplified and sub-cloned into the MICD4 vector. The MICD4-Flag-mDia2 WT expression construct was described previously. A flag-tagged constitutively active form of mDia2 with a DAD domain deletion (mDia2ΔDAD) (1–531 aa) and the HA-tagged mDia2 GBD/DID mutant (1–531 aa) were generated by inserting the PCR-amplified mutants into the MICD4 vector. The mouse Itgb2 expression construct pCMV-Itgb2 (Catalog #MG50359-UT) was purchased from Sino Biological Inc. (Beijing, China). The human ITGAM ORF was PCR amplified and sub-cloned into the MICD4 vector. The primer sequences are listed in Supplementary Table 1.

Flow cytometric assays. The preparation of single cell suspensions from mouse bone marrow and spleen tissue was described previously. In brief, bone marrow cells from femur and tibia were flushed out using a syringe with 30.5 G needle. The pelleted cells were resuspended in 1 ml FACS buffer (1× PBS containing 0.5% BSA and 2 mM EDTA). After centrifugation at 6000×g for 5 min, the cell pellets were lysed by incubation in 1× RBC lysis buffer (Catalog #00-4333-57, Thermo Fisher Scientific) for 5 min. The nucleated cells (white blood cells) were recovered and washed by centrifugation at 6000×g for 5 min before the cells were stained with anti-CD45, anti-CD11b, anti-Gr1, and anti-Kit (Catalog # 559971 BD Pharmingen) according to the manufacturer’s protocol. The stained cells were analyzed using a FACSCanto II (Becton, Dickinson and Company). The data were acquired on a FACSCanto II (Becton, Dickinson and Company) and analyzed with FlowJo software (Tree Star). The percentages of different cell populations were determined by manual gating. The survival curves in secondary bone marrow transplantations were plotted by using GraphPad software (GraphPad Software, Inc. La Jolla, CA).
The cells were then incubated with PE-Scal1(Ly-6A/E), PE-Cy7-CD117(c-Kit), APC-CD135, BV421-CD34, and PerCP-Cy5.5-CD16-CD32 for characterization of LT-HSC/ST-HSC/MPP and GMP/CMP/MEP populations. For analyses of SLAM-positive HSPCs, the cells were stained with PE-Scal1(Ly-6A/E), APC-eFlour780-CD48, and PerCP-Cy5.5-CD127 (IL-7ra). Expression of CXCR-4 and integrins in the hematopoietic progenitor cells was evaluated by co-staining lineage negative cells or c-Kit+ HSPCs with PE-Scal1(Ly-6A/E), PE-Cy7-CD117(c-Kit), and APC-CD184 (CXCR4)/APC-CD11a/CD11b/CD18. The information for antibodies is provided in Supplementary Table 1.

All staining was carried out at room temperature for 15–20 min. The samples were then washed with FACS buffer and kept on ice until FACS analysis. The absolute cell number was acquired by incorporating CountBright Absolute Counting Beads for flow cytometry (Catalogy® C36950, Thermo Fisher Scientific®). Gating of murine bone marrow HSPC subpopulations was performed as described previously, and was further illustrated in Supplementary Fig. 8. Flow cytometric analysis was performed with a BD LSRRFortessa cell analyzer, BD FACSCanto II, or BD LSRRFortessa X-20 flow analyzer, and further analyzed with FlowJo software (TreeStar Inc.).

**In vitro HSPC expansion**

c-Kit+ HSPCs from the bone marrow were purified using a mouse CD117 (c-Kit)-positive selection kit (STEMCELL Tech.) according to the manufacturer’s instructions. The purified c-Kit+ cells were further cultured in StemSpan serum-free expansion medium (SFEM) (Catalogy® #90650, STEMCELL Tech.) supplemented with 10 ng/ml IL-3 (Catalog #300-324P, GEMINI Bio-Products.), 10 ng/ml IL-6 (Catalog #300-327P, GEMINI Bio-Products.), 50 ng/ml SCF (Catalog # 300-348P, GEMINI Bio-Products.), and human LDL (1.6 mg/ml; Catalog #12-1000) (Catalog #02968, STEMCELL Tech.) for 24 h before transduction or further culture as indicated in the figure legends.

**Generation of retroviral particles and transduction of HSPCs.** To generate retroviral particles, HEK293T cells were seeded in 10 cm dishes at 6 x 10^6 for 16–18 h in high-glucose DMEM (Catalog #10-013-CM, CORNING®) containing 10% FBS (Catalog #900-108, GEMINI Bio-Products.), followed by co-transfection of 9 μg MDC4-based retroviral constructs and 4.5 μg of the packaging construct pC-Luci with TransIT-UTI transfection reagent (Mirus) according to the manufacturer’s protocol. Viral supernatants were collected 48 h after transfection and exchanged with fresh medium. After an additional 24 h, all virus-containing supernatants were pooled. The debris was removed by brief centrifugation. The virus supernatants were concentrated using a 100 kDa cutoff Amicon Ultra-15 Centrifugal Filter Unit (Catalog # UFC901024, EMD Millipore) through centrifugation at 4 °C following the manufacturer’s instruction. Retroviral infection of c-Kit+ HSPCs was performed by suspending the cells in freshly concentrated viral supernatants in the presence of 8 μg/ml polybrene (hexadimethrine bromide, Catalog #H9268, Sigma) and centrifuged at 900 x g for 90 min at 37 °C. After spin-infection, the infected supernatants were gently removed and the cells were incubated with fresh SFEM containing cytokines and further cultured for 36–48 h in vitro for further experiments as indicated for subsequent experiments.

**Quantitative real-time RT-PCR and RT-PCR.** RNA isolation, complementary DNA synthesis and quantitative real-time PCR were performed as previously described. Briefly, total RNAs from c-Kit+ HSPCs were extracted with TRIzol (Catalog #15596018, Invitrogen) according to the manufacturer’s protocol. cDNA was then reversely transcribed with 1 μg total RNAs by qScript cDNA supermix (Catalog #84034, Quanta Biosciences) as described by the manufacturer. Synthesized cDNA samples were amplified in a StepOnePlus Real-Time PCR System by using PerfeCTa SYBR Green QPCR FastMix with ROX (Catalog #95073-012, Quanta Biosciences) in triplicate. The default cycling conditions were 95 °C for 5 min and 40 cycles of 95 °C for 15 s, 60 °C for 60 s. Melting curve analyses were performed at the end of the reaction to confirm amplification of a single PCR product. Cycle threshold (Ct) values were calculated with StepOnePlus software (v2.3). The amplification efficiency of each pair of primer was determined by relative standard curve experiments. Relative target gene expression levels were further assayed by quantitative real-time RT-PCR with the comparative Ct (ΔΔCt) method and were normalized to 185 rRNA (eukaryotic 185 ribosomal RNA) levels in each sample. Results were expressed as mean ± S.D.

The RT-PCR procedure for detecting SRF isoforms was performed as previously described. Briefly, total cellular RNAs from FACS-sorted LK and LK cells were reverse transcribed by qScript cDNA SuperMix following the manufacturer’s protocol. PCR was performed in a separate tube with 5 μl of cDNA as a template. The 50 μl PCR reaction mix contained standard PCR buffer with 1.85 mM MgCl2, 0.2 mM deoxynucleoside triphosphates, sense and antisense primers (each 0.4 μM), and platinum Taq polymerase (Life Technologies). The cycling conditions included an initial 3 min 95 °C denaturation, followed by 50 cycles of denaturation for 15 s at 95 °C, annealing at 50 s for 55 s, and a 3 min extension at 72 °C. A 10 μl aliquot of reaction mix was fractionated on a 2% agarose gel, stained with ethidium bromide, and photographed. Negative controls lacked cDNA templates in the reaction mix. The primer sequences are listed in Supplementary Table 1.

**Adhesion assay.** c-Kit+ HSPCs purified from control or miD2-deficient mice were incubated with HSPC expansion medium (SFEM) containing cytokines for 3 days. The suspended cells were applied for adhesion assays with collagen (H-22)-coated or fibronectin (GG-22)-coated coverslip (Neuvis) for 1 h at 37 °C, or on poly-L-lysine (CORNING, RE834085)-coated coverslips for 15 min at 37 °C. After washing with PBS twice, the cells were fixed and stained with DAPI. For ICAM-1-binding assays, the coverslips were coated with recombiant mouse ICAM-1 (Gln28-Asn485) (Catalog# 796-IC, R&D) following the manufacturer’s instructions, and incubated with cells for 2 h at 37 °C. After washing with PBS, the cells were fixed and stained with DAPI, 5–10 random fields were selected in each coverslip with duplication for cell counting under an inverted fluorescent microscope at a magnification of 200x. The adhesion ability of indicated cells was normalized with respect to the control.

**In vitro trans-endothelial migration of HSPCs.** Murine EC (Catalog #C57-6221) and the complete culture medium (Cat# MI168) were purchased from Cell Bio- logics (Chicago, USA). EC (6 x 10^4) were seeded on the upper chamber of a Transwell system (Corning, USA) precoated with 0.1% gelatin for 72 h. Endothelial cell monolayer formation was visualized and examined with crystal violet staining. The endothelial cell complete medium was then removed and the cells were washed twice with PBS prior to loading HSPCs enriched in Lin− cells (6 x 10^4) in serum-free IMDM. After incubating for 4 h at 37 °C, cells that had migrated to the lower chamber with or without 200 ng/ml SFDF1a and 1% input cells were collected and used in a colony formation assay as described above except that erythropoietin was not added. The number of HSPCs that had migrated was calculated as the percentage of colony-forming cells (CFC) in the lower chamber normalized to CFC generated from input cells.

**Bone marrow transplantation.** Non-cBMT and cBMT were performed as described previously. Briefly, the recipient mice (8–9 weeks old) were pre-treated with 1 mg/kg of trastuzumab (Genentech) or 1 μg ubiquitin (a gift from Dr. John Appella, University of Pennsylvania) or 2000 U/ml polymyxin B, Sigma) for one week. The mice were then lethally irradiated (1000 rad) followed by retro-orbital injection of BMMC (2 x 10^6 for...
BMT, 2 × 10^6 plus an equal number of CD45.1+ BMMCs for cBMT or c-kit+ cells (1 × 10^6 for BMT, 1 × 10^6 mixed with an equal number of CD45.1+ competitors for cBMT). For serial transplantation, 2 × 10^6 donor BMMCs from primary transplant recipient mice were transplanted into lethally irradiated mice without otherwise indicated. All the recipient mice continued to receive water containing antibiotic for 3 weeks, after which they were given regular water. Flow cytometry of peripheral blood was performed to assess engraftment. Animal survival was monitored throughout the experimental period.

For the intrafemoral injection, mice were anesthetized by isoflurane inhalation. The left or right knee joint was sterilized through three rounds of 70% alcohol wash. Cells isolated from the bone marrow as described above were injected into the joint by puncture with a 22-gauge needle.

**Complete blood cell counts.** Peripheral blood samples were obtained by retro-orbital (RO) bleeding and stored in Greiner MiniCollect EDTA tubes containing K3EDTA (Catalog #450475). Complete blood cell counts were assayed using a Hemavet 950 instrument (Drew Scientific).

**Cell quiescence analysis.** Stem cell quiescence was profiled by pyronin Y and Ki67 staining. Briefly, HSPC-enriched lineage-negative cells from the bone marrow were labeled with PerCP-Cy5.5-ScA1 and APC-CD117 (c-Kit), washed and re-suspended in Iscove’s modified Dulbecco’s medium (IMDM; Catalog #12440-046, Thermo Fisher Scientific) containing 10 µg/ml Hoechst 33342. The cells were incubated for 45 min at 37 °C. Pyronin Y solution (100 µg/ml in PBS; Catalog #123159, Sigma) was then added directly to the cell suspension to a final concentration of 50 µg/ml for 10 min at room temperature. Prior to analysis, cells were subjected to antibody staining with PE-Sca1 and PE-Cy7 CD117 (c-Kit) for FACS analysis. Colonies formation assays were performed using nucleated cells from the peripheral blood. Colony numbers were scored using an inverted microscope and calculated on the basis of colony number per milliliter of peripheral blood. Cells from the bone marrow well were pipetted with 1× PBS, centrifuged, and re-suspended for FACS assay to determine the cell numbers. The data are presented as the number of cells derived from colonies per microliter of peripheral blood.

**Homing assay.** BMMCs (2 × 10^6) or bone marrow lineage negative cells (CD45.2+ , 2 × 10^6) from control or mdx2a-deficient mice were mixed with an equal number of CD45.1 competitive BMMCs or lineage-negative cells. The cells were then injected retro-orbitally into lethally irradiated WT recipient mice that were sacrificed at 24 or 48 h later for flow cytometry of BMMCs. Lineage-negative cells were harvested from the tibia and femur 12 h post injection for FACS analysis of c-Kit+ HSPCs. The population of CD45.1+ versus CD45.2+ cells was determined.

**Inmunofluorescent staining.** Sorted c-Kit+ HSPCs were suspended in serum-free IMDM or IMDM containing 10% FBS and plated onto poly-l-lysine-coated coverslips, followed by incubation at 37 °C in a humidified incubator for 10 min. The attached cells were washed with ice-cold PBS, fixed in 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. After rinsing three times in PBS, the cells were blocked with 3% BSA in PBS with 0.05% Triton X-100 for 1 h at room temperature. The cells were then washed and blocked with anti-rat serum overnight followed by staining with FITC-anti mouse/rat-Kit 67-monocondal antibody (SolA15) (Catalog #11-5698-82) and a Foxp3/transcription factor staining buffer set (Catalog #00-5523-00, eBioscience). In brief, after initial staining with PE-Sac1 (SolA15) (Catalog #11-5698-82) and a Foxp3/transcription factor staining buffer set (Catalog #00-5523-00, eBioscience), the cells were fixed with 30 min at room temperature. The cells were then washed and blocked with anti-rat serum overnight followed by staining with FITC-anti mouse/rat-Kit 67-monocondal antibody (SolA15, 1:1000; Catalog #11-5698-82) for 1 h in the dark at room temperature. Prior to analysis, cells were incubated with Hoechst 33342 for 30 min at room temperature.

**Site-directed mutagenesis or deletions.** To generate site directed and deletion mutants, PCR in a 20 µl reaction volume containing 10 ng of plasmid DNA template, 20 ng primer pairs and 0.5 U AccuPrime Pfx DNA polymerase in 1× reaction mix was performed. PCR amplification was initiated with 2 min at 95 °C to denature the template DNA, followed by 18 amplification cycles for 30 s each cycle, 55 °C for 1 min and 68 °C for 5–8 min depending on the template construct length (1 kV/min for Pfu DNA polymerase). The PCR products were treated with 0.5–1 µl of FastDigest Dpn1 (FD1703, Thermo Fisher Scientific) at 37 °C for 45 min and 8 µl of each PCR product was further analyzed by agarose gel electrophoresis. 5 µl PCR products generated above were transformed into E. coli competent cells. All site directed and deletion mutants were verified by DNA sequencing.

**Luciferase constructs with IsgaM and Isgb2 intronic regions.** Genomic DNA from wild type mouse tail was extracted by precipitation using two volumes of ethanol after Proteinase K (E00492, Thermo Fisher Scientific) digestion. Genomic DNA (5–10 µg) was used for PCR amplification with FastStart PCR Master Mix (Catalog #13000012, Thermo Fisher Scientific) or CloneAmp HiFi PCR Premix (Catalog #638500, Takara). Thermal cycling conditions were denaturation at 95 °C for 10 min, followed by 32 PCR cycles of 95 °C for 15 s, 65–67 °C for 30 s, and 72 °C for 1.5 min. The PCR products were treated with 0.5–1 µl of FastDigest Dpn1 (FD1703, Thermo Fisher Scientific) at 37 °C for 45 min and 8 µl of each PCR product was further analyzed by agarose gel electrophoresis. 5 µl PCR products generated above were transformed into E. coli competent cells. All site directed and deletion mutants were verified by DNA sequencing.
and calculated as the fold-change from empty vector. All the luciferase experiments were performed in duplicate, and the data are presented as mean ± SEM from 3 to 4 independent measurements using separate luciferase constructs from two or three different clones.

**Rapid measurement of sgRNA activity by luciferase reporter.** To establish a rapid and quantitative method to reliably evaluate genome-editing activities of various sgRNAs in mammalian cells, we built a luciferase activity-based reporter assay system. Although editing-based disruption of constitutively expressed fluorescence reporters, such as GFP and mRFP657 have been widely applied to test sgRNA activity, this approach involves a long detection period of 8–14 days. As such, decreases in fluorescence signal intensity do not occur immediately, even with the genetic disruption of the fluorescent gene locus because of the half-life of the remaining intact transcripts and turnover due to protein degradation.7–10 To overcome this delayed effect, we took advantage of the rapid response of the pGL4.24 luciferase construct (Promega), in which the PSEST sequence, a protein degradation signal from the C-terminal region of mouse ornithine decarboxylase, is incorporated into the synthetic firefly luc2 gene (luc2P). Specifically, sgRNAs were designed online using the CCTOP-CRISPR/Cas9 target online predictor or Design sgRNA online tool (Broad Institute). The sgRNAs were ligated into the sgRNA expression vector pBPK1520 (Addgene #56777) and pLKO5. sgRNA.ESF.mRFP657 (Addgene #57982). Potential sgRNA-targeting sites were selected and synthesized as gBlock DNA fragments (IDT), which were further PCR amplified and in-frame fused with the luc2P gene between the NcoI and ApaI sites by in-fusion cloning (Fig. 6a). To establish a Streptococcus pyogenes Cas9 overexpression stable cell line, HEK293T cells were transfected with pLenti-Cas9-BSD (Addgene #52962) lentivirus and subsequently selected with 10 μg/ml blastidicin (Thermo Fisher Scientific, R21001) to obtain single clones. HEK293T–48 monoclonal cells showed strong FLAG-Cas9-NLS expression and consistent efficiency and yield as a rapid mammalian genome-editing reporter system for all subsequent assays. The newly designed target gene name mimics luciferase construct (100 ng per well) was transiently transfected into HEK293T–48 cells together with various sgRNA vectors (300–400 ng per well) in the presence of pRL-TK (1 ng per well). Luciferase activity was measured 24 h post-transfection. When screened against sgRNA targeting SRE sites in the Acta2 intronic region but not the exonic sequences, this inhibitory effect was depressed by adding DNA sequences encoding 3x(GSSSS) amino acids between the intrinsic target sites and the luc2P gene11 (Supplementary Fig. 6c, d).

**ChIP assay.** Purified c-KIT– bone marrow HSPCs were incubated with or without 10% FBS in IMDM for 30 min and then collected in formaldehyde. ChIP assays were performed using the Pierce Magnetic ChIP Kit (Catalog #26157, Thermo Fisher Scientific) according to the manufacturer’s protocol. Briefly, chromatin was incubated with anti-SRF antibody (sc-390324, Santa Cruz), or anti-GFP (B2, Catalog #sc-9996, Santa Cruz) antibody and then incubated with anti-SRF antibody (2C5, Catalog #61386, Active Motif), anti-MAL antibody (e, h, j, 5a, c, e, f, b, e, h, i) and Supplementary Figs. 1a, d, 2a–d, g, 3a–c, f, b–d, 5b, c, a, b, d, and 7a–f available as a Source Data file. Other data that support the findings of this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Competing interests
The authors declare no competing interests.

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