The Microtubule Plus-End Tracking Protein CLASP2 Is Required for Hematopoiesis and Hematopoietic Stem Cell Maintenance

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SUMMARY

Mammalian CLASPs are microtubule plus-end tracking proteins whose essential function as regulators of microtubule behavior has been studied mainly in cultured cells. We show here that absence of murine CLASP2 in vivo results in thrombocytopenia, progressive anemia, and pancytopenia, due to defects in megakaryopoiesis, in erythropoiesis, and in the maintenance of hematopoietic stem cell activity. Furthermore, microtubule stability and organization are affected upon attachment of Clasp2 knockout hematopoietic stem-cell-enriched populations, and these cells do not home efficiently toward their bone marrow niche. Strikingly, CLASP2-deficient hematopoietic stem cells contain severely reduced mRNA levels of c-Mpl, which encodes the thrombopoietin receptor, an essential factor for megakaryopoiesis and hematopoietic stem cell maintenance. Our data suggest that thrombopoietin signaling is impaired in Clasp2 knockout mice. We propose that the CLASP2-mediated stabilization of microtubules is required for proper attachment, homing, and maintenance of hematopoietic stem cells and that this is necessary to sustain c-Mpl transcription.

INTRODUCTION

Cells of the blood are generated in a complex process called hematopoiesis. Production begins with hematopoietic stem cells (HSCs), which are present in a very limited number in the adult bone marrow. HSC maintenance is achieved by self-renewal, whereas HSC differentiation eventually generates cells of all the blood lineages. Attachment, migration, and (a)symmetric cell division are important for the maintenance, proliferation, and differentiation of all hematopoietic cells, including HSCs. These processes are regulated by the microtubule (MT) cytoskeleton. MTs are dynamic polymers assembled from heterodimers of α- and β-tubulin. MTs perform many of their cellular tasks by changing their organization and stability in response to the needs of the cell. This process is largely controlled by MT-associated proteins. Some of these factors are localized specifically at the ends of growing MTs and are called “plus-end tracking proteins,” or +TIPs (for review, see Galjart, 2010).

Mammalian CLASP1 and CLASP2 are +TIPs that can stabilize MTs in specific regions of the cell, thereby regulating both mitosis and interphase. In motile fibroblasts, for example, CLASP2 acts downstream of PI3K and GSK-3β to enhance MT stabilization at the leading edge and support directed motility (Akhmanova et al., 2001; Drabek et al., 2006). The C terminus of CLASP2 is required for association with the cell cortex, through LL5β and ELKS (Lansbergen et al., 2006), proteins that might form a PI3K-regulated cortical platform to which CLASPs attach distal MT ends. Cortex localization and clustering of LL5β...
Figure 1. Hemorrhages, Pancytopenia, and Erythroid Defects in Clasp2 KO Mice

(A) Western blot analysis. Protein extracts from the bone marrow of WT and Clasp2 KO mice were analyzed on western blot using anti-CLASP2 (12H2) and anti-CLIP-170 (#2360) antisera.

(B and C) Growth curves. Body weights of WT, heterozygous (HET), and homozygous Clasp2 KO mice are shown at different time points. Groups consisted of 5 KO, 10 HET, and 9 WT males (B) and 4 KO, 14 HET, and 9 WT females (C).

(D) Hemorrhages in Clasp2 KO mice. Ovaries from WT and Clasp2 KO mice were isolated. In both KO ovaries, severe hemorrhages are present.

(E and F) WBC and RBC counts. Blood was isolated from 12 WT and 16 Clasp2 KO mice.

(G) Reticulocyte counts. Blood from three WT and three Clasp2 KO mice was deposited on glass slides. Approximately 2,000 RBCs were counted per mouse, and the percentage of reticulocytes was measured.
in epithelial cells are regulated by integrins and the cell-adhesion molecule laminin (Hotta et al., 2010), providing a link among cell adhesion, integrin signaling, CLASPs, and MTs.

To examine the in vivo role of CLASP2, we generated Clasp2 knockout (KO) mice. These mice display multiple defects, including problems in megakaryopoiesis, erythropoiesis, and the maintenance of the HSC bone marrow pool. We show that the MT network is affected in CLASP2-deficient HSC-enriched bone marrow cells and that these cells do not home efficiently toward their niche. Interestingly, expression of c-Mpl, which encodes the thrombopoietin (TPO) receptor (Kaushansky, 2009), is reduced in CLASP2-deficient HSCs. Our data reveal a link between the mechanical action of CLASP2 at MT ends and the expression of a hematopoietic factor that is important for megakaryocyte development and HSC maintenance.

**RESULTS**

**Pancytopenia in Clasp2 KO Mice**

We disrupted the murine Clasp2 gene (Figures S1A and S1B) and showed abrogation of CLASP2 expression (Figures 1A and S1). Matings between heterozygous Clasp2 KO mice yielded less homozygous offspring than expected (29% wild-type [WT], 50% heterozygous, and 22% homozygous Clasp2 KO mice in 729 mice counted). The body weight of homozygous Clasp2 KO mice was approximately 30% lower than that of WT and heterozygous littermates (Figures 1B and 1C). Patho-anatomic examination of adult Clasp2 KO mice revealed severe anomalies in male and female reproductive organs but no gross alterations in other tissues (data not shown). These results indicate that CLASP2 plays a role in germ cell development as well as in multiple somatic tissues.

We observed hemorrhages in different Clasp2 KO organs, including the reproductive system (Figures S1D and S1E) and stomach (in 67% of the mice). Petechiae, small red spots caused by a minor hemorrhage, were found in the brain, bladder, and lymph nodes (in 20%, 17%, and 11% of the KOs, respectively). Furthermore, white blood cell (WBC) and red blood cell (RBC) numbers were reduced in Clasp2 KO blood (Figures 1E and 1F), as were hematocrit and hemoglobin levels (data not shown). By contrast, we detected more reticulocytes in Clasp2 KOs (Figure 1G), indicating that immature RBCs are extruded into the blood to compensate for the erythrocyte deficiency.

Platelets were also reduced in Clasp2 KOs (Figure 1H). Because a reduction in platelet number is by itself not sufficient to explain a bleeding phenotype, we analyzed whether defects in the endothelium that lines the blood vessels could explain the internal bleedings, but we detected no obvious endothelial damage in hematoxylin/eosin-stained sections of Clasp2 KO mice (data not shown). Because platelets need to adhere in order to form a clot, we examined platelet adhesion to collagen under physiological shear rate. Strikingly, adhesion was almost completely inhibited in CLASP2-deficient platelets (Figure 1I), indicating that this underlies the hemorrhages in Clasp2 KO mice. Taken together, our data reveal a severe pancytopenia in Clasp2 KO mice.

TPO is a major regulator of platelet homeostasis in vivo (Kaushansky, 2009), whereas erythropoietin (EPO) controls the levels of RBCs (Richmond et al., 2005). TPO values were about four times higher, and EPO levels were even more increased in Clasp2 KO mice compared to WT littermates (Figures 1J and 1K, respectively). These results indicate that the thrombocytopения and anemia are sensed by the Clasp2 KO mice; however, despite elevated TPO and EPO levels, platelet and RBC numbers were not restored to normal values. Furthermore, in addition to reduced WBC, RBC, and platelet numbers in the blood, we also observed fewer nucleated cells in the bone marrow of Clasp2 KO mice (Figure 1L). We therefore investigated the various hematopoietic lineages in Clasp2 KO mice in more detail.

**CLASP2 Is Required for Erythropoiesis**

During erythropoiesis, committed erythroid progenitors (burst-forming unit erythroid [BFU-E] and colony-forming unit erythroid [CFU-E]) differentiate toward the erythroblast stage, which develops further into enucleated reticulocytes and, finally, into mature erythrocytes, or RBCs. In case of anemia, erythroid progenitors relocate to the spleen and commence stress erythropoiesis. As a result, the spleen increases in size. Clonogenic culture assays showed reduction of bone marrow-derived BFU-E and CFU-E colonies in the absence of CLASP2 (Figure S1F). By contrast, in the spleen, a slight increase in the number of these colonies was observed (Figure S1G), indicative of stress erythropoiesis.

Further analysis of the erythroid system revealed a significant decrease in early c-kit+CD71+ erythroblasts in the Clasp2 KO bone marrow, whereas the amount of late-stage CD71+ TER119+ erythroblasts was similar to WT (Figure 1M). By contrast, in the Clasp2 KO spleens, both early- and late-stage erythroblasts were increased (Figure 1N). Taken together, our data suggest that a CLASP2 deficiency limits erythroid...
differentiation in the bone marrow. This induces stress erythropoiesis in the spleen, which is consistent with an increased spleen/body weight ratio in Clasp2 KO mice (Figure S1E). Stress erythropoiesis is, however, not sufficient to compensate for the RBC loss in the blood (Figure 1F), which explains the highly increased EPO levels (Figure 1K).

Flow cytometry analysis showed increased amounts of CD71, the transferrin receptor, on the cell surface of CLASP2-deficient erythroblasts (Figures 1M–1O). Strikingly, CD71 expression was higher in 12-week-old than in 6-week-old Clasp2 KO mice (Figure 1O). Because CD71 is a target of EPO (Sivertsen et al., 2006), the age-dependent increase in CD71 expression in Clasp2 KO mice indicates that EPO levels also increase with age. EPO levels, in turn, correlate with severity of anemia. We therefore propose that lack of CLASP2 results in progressive anemia.

CLASP2 Is Required for Megakaryopoiesis

Histological sections suggested a deficit of megakaryocytes, the cells that give rise to platelets, in Clasp2 KO bone marrow (Figures 2A and 2B). Flow cytometry analysis suggested impairment in all stages of megakaryopoiesis (Figures 2C and S2A). Megakaryocytes acquire high DNA ploidy levels by endomitosis, allowing them to grow very large and form many platelets from a single megakaryocyte. DNA staining indicated that Clasp2 KO megakaryocytes skew toward a lower ploidy status (Figure 2D). Analysis in fetal liver-derived megakaryocyte cultures showed that Clasp2 KO megakaryocytes were smaller than WT (Figures 2E and 2F). In addition, in bone marrow-derived cultures, 10-fold fewer megakaryocytes developed (Figure 2G), and the fraction of KO megakaryocytes with >8N DNA content was lower (Figure 2H). Furthermore, we observed a decrease in Clasp2 KO megakaryocyte progenitor cells (colony-forming unit megakaryocyte [CFU-MK]) in clonogenic assays (Figure 2I). Thus, CLASP2 deficiency affects megakaryocytes at the level of progenitors and at later stages. The block in maturation leads to fewer and smaller megakaryocytes with reduced DNA content. Absence of CLASP2 did not appear to impede (pro)platelet formation (Figures S2B–S2E), indicating that CLASP2 is not involved in the final stages of megakaryopoiesis. Platelet activation upon thrombin stimulation was also not affected by the lack of CLASP2 (Figures S2F–S2M).

Because endomitosis requires the MT-based movement of chromosomes, we asked if CLASP2 localizes to the mitotic spindle machinery. Immunofluorescence analysis showed that in WT megakaryocytes from 6 day cultures, CLASP2 colocalizes with the CREST kinetochore marker (Figures 2J–2M). Thus, CLASP2 might have a role in endomitosis as a kinetochore protein. Although this is a novel finding, it is consistent with a role for CLASP2 at kinetochores in fibroblast mitosis (Pereira et al., 2006).

CLASP2 Is Required for HSC Maintenance and Homing

Flow cytometry analysis in HSC-enriched and early progenitor populations revealed a reduction in all fractions in Clasp2 KO mice (Figures 3A and S3A–S3C), including Lin−/Kit+ /Sca1+ (LSK) cells, which contain both long- and short-term HSCs, and CD48−/CD150− LSK cells, which are further enriched for long-term HSCs (Kiel et al., 2005). Thus, a deficiency of CLASP2 affects HSCs and early progenitors.

To examine whether the stem cell defect in Clasp2 KO mice is due to malfunctioning HSCs themselves or to an abnormal stromal environment, we performed transplantation assays. We injected bone marrow cells derived from Clasp2 KO mice and WT littermates into sublethally irradiated recipients and examined donor HSC progeny in the bone marrow of recipient mice after short (i.e., 1 month) and long-term (i.e., 4 months) reconstitution. Bone marrow cells from the Clasp2 KO mice failed to fully reconstitute irradiated recipient mice even with the highest dose of cells injected (Figure 3B). By contrast, bone marrow cells from WT donors reconstituted efficiently, even when few cells were injected (Figure 3B). These data reveal a cell-autonomous HSC defect in Clasp2 KO mice.

To examine whether CLASP2 plays a role in HSC homing in vivo, we isolated bone marrow cells from WT and Clasp2 KO mice, loaded these with the fluorescent compound CFSE, and injected cells into sublethally irradiated recipients. Fifteen hours after injection, we examined the number of CFSE− LSK cells in the bone marrow of the recipient mice and compared this value to the number of original CFSE+ LSK cells injected. The efficiency with which CLASP2-deficient LSK cells homed to their niche in recipient bone marrow was approximately half of that of WT cells (Figure 3C). We conclude that, akin to its role in persistent motility in fibroblasts (Drabek et al., 2006), CLASP2 is involved in the homing of LSK cells in vivo.

CLASP2 Deficiency Affects c-Mpl Transcription

To investigate whether the hematopoietic phenotype in CLASP2-deficient mice might be explained by a differential expression of CLASP1 and CLASP2, we examined Clasp1 and Clasp2 mRNA levels in different bone marrow fractions by RT-PCR. This revealed that Clasps are expressed at similar levels throughout the hematopoietic system (Figure S3D). Thus, CLASP1 and CLASP2 are not redundant in vivo.

We next investigated at a genome-wide level whether lack of CLASP2 affects distinct genes and/or signaling cascades. Next-generation sequencing of LSK-derived mRNA showed that many mRNAs, including Actin, Clasp1, and Slam1 (CD150), were not affected by a lack of CLASP2 (Figures 3D and S3F; Table S1). However, the levels of Meis1, which encodes an important transcription factor for megakaryopoiesis and HSC survival (Hisa et al., 2004; Simsek et al., 2010), and of c-Mpl, which encodes the TPO receptor (Kaushansky, 2009), were severely reduced in the absence of CLASP2 (Figures 3D and S3F). Real-time PCR confirmed the reduction in Meis1 (Figure S3E).

One reason for the reduced expression of Meis1 and c-Mpl in LSK cells could be that long-term HSCs are underrepresented in the Clasp2 KO. We therefore also sequenced CD150+ LSK fractions, which are highly enriched for HSCs (Kiel et al., 2005). Whereas Meis1 was only moderately affected in Clasp2 KO CD150+ LSK cells, c-Mpl expression was still severely decreased (Figure 3E). Moreover, the expression of Prdm16, an HSC marker (Forsberg et al., 2010) and target of c-Mpl in HSCs (Heckl et al., 2011), was also downregulated (Figure 3E). These results suggest that absence of CLASP2 leads to aberrant c-Mpl transcription and c-Mpl signaling.
Figure 2. Megakaryocyte Defects in Clasp2 KO Mice

(A and B) Hematoxylin and eosin-stained sections of the bone marrow. (A) WT and (B) Clasp2 KO bone marrow is shown. Megakaryocytes are indicated with arrows. Scale bar, 100 μm.

(C) Megakaryocyte differentiation in the bone marrow. Consecutive stages in megakaryocyte differentiation were analyzed by flow cytometry. MK-blast, megakaryoblast; MK-pro, megakaryocyte progenitor; MK1, MK2, and MK3, early and mature megakaryocytes. SD is indicated.

(D) DNA content in bone marrow megakaryocytes. Ploidy levels measured by flow cytometry in two WT and two Clasp2 KO mice and expressed as percentages of total number of CD41+ cells are presented. Average values are shown.

(E–H) Liquid megakaryocyte cultures. Phase-contrast images (E and F), megakaryocyte counts (G), and DNA content analysis (H) of fetal liver (E and F) and adult (G and H) megakaryocyte cultures from WT and Clasp2 KO are presented. (G) SEM is indicated; t test reveals significant difference. (H) Ploidy levels in five WT and seven KO cultures expressed as percentages of total number of observed cells are demonstrated. Scale bar, 50 μm.

(I) CFU-MK progenitor assay. Number of colonies (per 50,000 cells seeded) counted after 10 days in culture is shown (SD is indicated; t test reveals significant difference).

(J–M) Localization of CLASP2 in megakaryocytes. WT cells were stained with CLASP2 antibodies (green), the kinetochore marker CREST6 (red), and DAPI (blue). Kinetochore association of CLASP2 is indicated by arrows. Scale bar, 3 μm.

See also Figure S2.
CLASP2 Organizes the MT Network upon Cell Attachment

CLASP2 selectively regulates MT stabilization in motile fibroblasts (Akhmanova et al., 2001). Because fibroblast adherence also results in selective MT stabilization (Palazzo et al., 2004), we asked whether CLASP2 is involved in this process too. Indeed, CLASP2-deficient fibroblasts plated on a fibronectin-coated matrix contained less stable MTs compared to WT cells (Figure S4).

Next, we isolated WT and CLASP2-deficient HSC-enriched (LSK) cells, allowed them to attach, and examined the MT network and associated organelles using specific antibodies. EB1 staining revealed ample-growing MTs, which originated at the centrosome and circumnavigated the nucleus (Figures 4A–4C). No obvious difference was observed in the number of EB1 dots in WT and CLASP2-deficient HSC-enriched cells. Examination of the MT network using anti-α-tubulin antibodies revealed a cage-like organization in most of the WT cells, with MTs originating at the centrosome and surrounding the nucleus (Figures 4D and 4E). The Golgi network, as observed with anti-GM130 antibodies, was clustered around the centrosome; its organization was similar in WT and Clasp2 KO HSC-enriched cells (Figures 4F and 4G).

In contrast to the Golgi apparatus, the distribution of stable MTs was significantly different in CLASP2-deficient HSC-enriched cells compared to WT cells (Figures 4H–4L). MT organization was also affected, i.e., approximately half of the CLASP2-deficient HSC-enriched cells contained a cage-like MT organization compared to WT cells (Figure 4M). Combined, our data suggest that CLASP2 is required for cell attachment and the subsequent stabilization of the MT network, as well as its organization, both in freshly isolated HSC-enriched cells as well as in cultured MEFs.

DISCUSSION

We show here that CLASP2 is essential for mouse hematopoiesis, acting at multiple steps in erythroid, megakaryocytic, lymphoid, and myeloid differentiation. Shortages in RBCs and platelets in Clasp2 KO mice lead to increased EPO and TPO levels and induce a stress response. However, these reactions are insufficient, and thus, an enhanced replenishment by HSCs is required. Because CLASP2 is also necessary, in a cell-autonomous manner, for HSC maintenance, the stem cell pool becomes exhausted in Clasp2 KO mice, explaining the progressive pancytopenia. This phenotype resembles that of patients with congenital amegakaryocytic thrombocytopenia, which starts as a severe thrombocytopenia at birth and then rapidly progresses to pancytopenia (Ballmaier and Germeshausen, 2009). Most patients carry a mutation in the c-Mpl gene.
Strikingly, one of the most downregulated genes in Clasp2 KO HSC-enriched fractions is c-Mpl. Thus, a major molecular mechanism underlying the HSC maintenance defect in Clasp2 KO mice might be impaired TPO signaling through c-Mpl.

How is it possible that CLASP1, which is similar to CLASP2 and also expressed in the hematopoietic system, cannot compensate for a CLASP2 deficiency? One explanation is that CLASP levels are critical, such that in the absence of CLASP2, there is not enough CLASP1 to compensate for a lack of CLASP2. Alternatively, CLASP2 might be recruited by signaling pathways that do not act on CLASP1, and vice versa. Recent work has indeed shown that CLASP1, and not CLASP2, is important for maintaining spindle position and a correct cell division axis in human cells (Samora et al., 2011). CLASPs may therefore coexist in cells, but distinct cascades, acting through phosphorylation (Kumar et al., 2009), might selectively affect their activity toward MTs.

Our data show that despite their round shape and small cytoplasm attached HSC-enriched cells are polarized, with centrosome and Golgi located at one side of the nucleus and the plus ends of MTs emanating toward the other side. Both in these cells and in fibroblasts, CLASP2 stabilizes and organizes the MT network. Furthermore, CLASP2 is required for the proper attachment of fibroblasts, HSC-enriched cells, and platelets, indicating a general role for this protein in cell attachment. It remains to be shown whether an integrin-LL5-CLASP2-MT connection is operative in these systems as it is in epithelial cells (Hotta et al., 2010). We hypothesize that in the absence of CLASP2, long-term HSCs do not recognize their bone marrow niche anymore because of faulty attachment and/or migration, turn off c-Mpl transcription due to deregulated signaling and thereby lose stem cell potential.

EXPERIMENTAL PROCEDURES

Miscellaneous Assays
We have obtained all the necessary permissions from local and national review boards for the mice used in our research. Gene targeting and immunohistochemistry techniques (Hoogenraad et al., 2002), generation of CLASP2-deficient fibroblasts and immunofluorescent staining assays (Drabek et al., 2006), and standard molecular biology techniques (Sambrook et al., 1989) have been described. See Extended Experimental Procedures for details.

Hematological Analyses
Hematological assays were performed in peripheral blood, platelets, bone marrow, spleen, thymus, and lymph nodes. Where needed, cells were sorted by flow cytometry and analyzed with FlowJo software. For in vivo HSC analysis, total bone marrow cells from adult WT and Clasp2 KO male mice were intravenously injected into irradiated recipients. After 1 and 4 months, donor chimerism was analyzed by semiquantitative PCR on peripheral blood. For
the in vivo homing assay, bone marrow cells from WT and Clasp2 KO mice were labeled with CFSE, analyzed by flow cytometry to estimate the percentage of CFSE<sup>+</sup> LSK cells (time point 0 hr), and intravenously injected into irradiated recipients. After injection (15 hr), bone marrow cells were collected, and the number of CFSE<sup>+</sup> LSK cells was again determined. The fraction of LSK cells homing into the bone marrow was calculated by dividing the number of donor CFSE<sup>+</sup> LSK cells at 15 hr by the number of CFSE<sup>+</sup> LSK cells injected at 0 hr. See Extended Experimental Procedures for details.

**Genome-wide Transcriptome Analyses**

RNA was isolated from LSK and CD150<sup>+</sup> LSK fractions from WT and Clasp2 KO mice and sequenced using the Illumina platform. See Extended Experimental Procedures for further details.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.08.040.

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EXTENDED EXPERIMENTAL PROCEDURES

Antibodies and Reagents
For immunofluorescence studies and Western blot analysis, we used monoclonal anti-CLASP1 and –2 antibodies (Maffini et al., 2009), rabbit polyclonal antibodies #2358 against CLASP2 (Akhmanova et al., 2001), antiserum #2360 against CLIP-170 (Coquelle et al., 2002), antisera #2221 against CLIP-115 and –170, antiserum #2238 against CLIP-115 (Hoogenraad et al., 2002), and anti-EB3 antiseras (Stepanova et al., 2003). The anti-CREST6 antibodies were a kind gift of Dr. Y. Mimori-Kiyosue (KAN Research Institute, Kyoto, Japan). The following antibodies were purchased: anti-GFP antibodies (Abcam and Santa Cruz, Roche), mouse monoclonal antibodies against α-tubulin and acetylated alpha-tubulin (Sigma), and actin (Chemicon). Secondary antisera were alkaline phosphatase-labeled anti-rabbit and anti-mouse antibodies (Sigma), and FITC-conjugated goat anti-rabbit IgG and AlexaS94-conjugated goat anti-human antiseras (Molecular Probes).

For flow cytometry analysis we used the following antibodies: Ter119 Pacific Blue or PerCP-Cy5.5 conjugated (eBiosciences), CD41 PE, CD71 PE conjugated or biotinylated, c-kit APC or APC-Cy7 conjugated, Lineage cocktail APC conjugated, CD3e-biotin, B220-biotin, CD11b-biotin, Gr1-biotin, CD16/CD32 PE conjugated, CD127 biotin, CD34 Pacific Blue, Alexa Fluor 647 (eBiosciences) or PerCP-Cy5.5, Sca1 PE-Cy7, streptavidin APC-Cy7, PE-Cy5, PerCP-Cy5.5 or Pacific Blue (Invitrogen) conjugated. Antibodies were from BD Biosciences unless otherwise indicated. Hoechst 33342 (Invitrogen) was used to stain dead cells.

Generation of Clasp2-Deficient Mice and MEFs
Targeting techniques and the procedures for selection of ES cells and generation of KO mice (Akhmanova et al., 2005; Hoogenraad et al., 2002) have been described. Targeting efficiency in ES cells was very low (1/800). The positive ES cell clone contained a correct karyotype and was injected into blastocysts. One of four chimeras gave germline transmission and was mated into the C57BL/6 background to generate inbred Clasp2 heterozygous and homozygous KO mice. The generation of CLASP2-deficient MEFs has been described (Drabek et al., 2006).

Fibroblast Adhesion Assay
MEF adhesion experiments were essentially carried out as published (Palazzo et al., 2004). For IF analysis of the cell adhesion of MEFs, coverslips were coated with 25 μg/ml fibronectin or poly-L-ornithin (Invitrogen) and cells were examined 90 min after adhesion. Attachment on poly-L-ornithin does not lead to stable MT formation, neither in WT fibroblasts nor in CLASP2-deficient cells (data not shown).

To test adhesion capacity, a colorimetric assay was used. Briefly, trypsinized and resuspended cells were plated in 96-well plates coated with fibronectin of poly-L-ornithin (30,000 cells per well). Cells were fixed 90 min after adhesion, washed and stained with Crystal Violet (0.5%) for 10 min. Plates were washed with water and air-dried. Absorbance was read at 570 nm using a plate reader.

Immunofluorescence Analysis
For IF analysis of megakaryocytes, cells were cultured under the conditions described below. After 6 days of culture cells were centrifuged onto poly-L-lysine-coated coverslips at 400 rpm for 5 min, fixed for 15 min in ice-cold methanol/1mM EGTA and permeabilized with 0.5% Triton X-100. After blocking with 1% bovine serum albumin (BSA) in PBS, slides were incubated with a 1:300 dilution of primary antibodies, for one hour at room temperature. After washing, cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:100) and/or AlexaS94-conjugated goat anti-human (1:300) antibodies, again for one hour at room temperature. Cells were counterstained with DAPI to visualize nuclear DNA. Controls were processed identically, except for the omission of the primary antibody, and did not give a detectable signal (data not shown).

For IF analysis of HSC-enriched cells, bone marrow single cell suspensions from Clasp2 KO mice and WT littermates were stained with Lineage cocktail APC, c-kit APC-Cy7 and Sca1 PE-Cy7 conjugated antibodies. The HSC-enriched cells were sorted in the FACSaria (BD Biosciences). Cells were concentrated by centrifugation and then spotted in a small volume (~10 μl) onto poly-L-lysine (Invitrogen) coated coverslips. Cells were allowed to adhere to the glass at 37 °C for ~30 min in StemPro®-34 SFM supplemented with B-27 Serum-Free Supplement and 1% Penicillin/Streptomycin/L-glutamine solution (Invitrogen). Cells were fixed and incubated with antibodies as described previously (Drabek et al., 2006). IF image acquisition was carried out as described (Akhmanova et al., 2005; Boisset et al., 2010). In Figures 4L and 4M, the fluorescence intensity (FI) of maximum intensity projections (MIPs) of cells, stained with anti-acetylated tubulin antibodies (which represents the amount of stable MTs) was quantified by placing exactly fitting ROIs around the cells and measuring the average FI (11 WT and 12 KO cells analyzed). Note that the difference between WT and KO is actually underestimated because in the WT MIP the FI is saturated in many areas of the cell (due to the stacking of the images), whereas this is not the case in KO cells. To quantify MT organization, cells with a “cage-like” MT network (see Figures 4D, 4E, and 4F), as identified with antibodies against tyrosinated tubulin and acetylated tubulin, were quantified in different microscopic slides containing WT (WT) or Clasp2 KO HSC-enriched cells (>300 WT and KO cells analyzed).

Hematological Analysis
Peripheral blood samples were taken from the retro-orbital venous plexus using EDTA-coated tubes. Blood counts were determined with a Vet’ABC counter (SCIL, Viernheim, Germany).
The analysis of erythropoietin (EPO) levels in plasma was performed using the Quantikine mouse/rat EPO immunoassay (BD Biosciences; Franklin Lakes, NJ). Thrombopoietin (TPO) levels were determined in serum by ELISA (Quantikine Mouse TPO Immunoassay; R&D Systems), according to the manufacturer’s instructions. Serum levels from each mouse were measured in duplicate. Values were imported into Aabel (Gigawiz) for graphical representation and statistical analysis.

Colonies forming unit- and burst forming unit-erythroid (CFUe and BFUe) assays were performed by culturing 2x10^5 cells per 35mm dish in methycellulose medium (MethoCult M3234, Stem Cell Technologies, Vancouver, Canada) supplemented with 4U/ml human recombinant EPO, (Janssen-Cilag, Issy-les-Moulineaux, France), 100ng/ml murine recombinant stem cell factor (SCF), 20 μg/ml iron-saturated human transferrin, 2x10^{-4} M hemin (Sigma) and 1% Penicillin/Streptomycin/L-glutamine solution (Invitrogen, Gaithersburg MD). Colony numbers were determined after 3 days (CFUe) or 8 days (BFUe) of culture on triplicate dishes.

Megakaryocytes were cultured from 13.5 day fetal livers in DMEM (GIBCO), supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% tissue culture supernatant from a murine TPO producer cell line (Villeval et al., 1997). After 3 days of culture, megakaryocytes were enriched on a BSA gradient as described previously (Italiano et al., 1999). Megakaryocytes from three WT and three Clasp2KO mice were counted after 6 days of culture in at least 5 microscopic fields of each culture. For proplatelet formation analysis, cultures were analyzed on day 5 by phase-contrast microscopy.

For the megakaryocyte progenitor cell assay (CFU-MK assay) bone marrow cells from 2 WT and 2 Clasp2KO mice were collected by flushing femurs and tibias of Clasp2KO or WT mice with Iscove’s DM containing 2% fetal bovine serum. Serum-free collagen assays were performed with a MegaCult TM -C kit (StemCell Technologies Inc.; Vancouver, Canada). A combination of human recombinant growth factors was used as a source of megakaryocyte (MK) colony-stimulating activity: 1.1 mg/ml collagen, 1% bovine serum albumin (BSA), 10 g/ml bovine pancreatic insulin, 200 g/ml iron-saturated human transferrin, 50 ng/ml thrombopoietin, 10 ng/ml interleukin 6, and 10 ng/ml interleukin 3. Cultures were prepared according to the manufacturer’s instructions. The final culture mixture of 1.5 ml was dispensed into the two wells (0.75 ml each) of a chamber slide and incubated in humidified 5% CO2 incubator at 37°C. After 10 days of incubation, slides were fixed in cold acetone (15 min), stained, and CFU-MK colonies were counted. CFU-MKs were identified by the detection of acetylcholinesterase activity of megakaryocytes. A CFU-MK colony was defined as a cluster of three or more MK cells detected by light microscopy.

To measure the DNA content of cultured megakaryocytes, individual single-cell suspensions were made from bone marrow harvested from the femurs of 5 WT and 7 Clasp2KO mice. Cells were cultured using the conditions described above. After the second day of culture, megakaryocytes were purified on a BSA gradient, cultured for one more day, and prepared for flow cytometry analysis. In short, harvested cells were fixed in ice-cold 70% ethanol for 48 hr, centrifuged and resuspended in PBS. Cells were subsequently stained with propidium iodide (PI) in a triton/RNase solution for 30 min at room temperature, and the DNA content was measured using a FACScan flow cytometer (Becton Dickinson). The proportion of cells in each ploidy class was determined by integrating the area under each peak. Lymphocytes from the spleen were used as 2n/4n control.

To measure the DNA content of freshly isolated bone marrow megakaryocytes, mouse bone marrow single cell suspensions were incubated with anti-CD41-PE (BD PharMingen, San Diego) for 1 hr on ice in PBS/1%BSA. Cells were then washed with 10 volumes PBS/1%BSA and spun at 1000 rpm for 5 min. Cells were resuspended in Cytofix/Cytoperm solution (Cytofix/Cytoperm, BD Biosciences) and incubated 30 min on ice. Cells were spun at 1000 rpm for 5 min, resuspended in 80% EtOH and resuspended in PBS containing 50 μg/ml Hoechst 33342, 0.1% Triton X-100 and 50 μg/mL RNase and incubated for 1hr at RT. Samples were analyzed on a flow cytometer (LSR-II, BD Biosciences) and analyzed with FlowJo software. Megakaryocytes were identified as CD41-positive cells and Hoechst 33342 staining intensity was used as a measure of DNA ploidy.

Platelet Adhesion and Morphology
For platelet adhesion assays, whole blood was perfused over collagen-coated (Horm-Collagen type I, 100 μg/ml) slides at a shear rate of 13000±1. Whole blood volume corresponding to the same circulating platelet number was perfused, after which adhered platelets were washed with PBS for 5 min. Platelets were then labeled with CD41 PE or CD61 FITC conjugated antibodies and photos were taken at 200x magnification with an EVOS microscope. Coverage (fluorescence) was quantified with ImageJ software.

For morphology assays platelets were separated from whole blood by sequential centrifugation (Schwer et al., 2001). Platelets from the resulting platelet rich plasma (PRP) were allowed to rest for 30 min at 37°C, before fixation. To examine cell shape, platelets were fixed with an equal volume of 8% PFA for 20 min and centrifuged onto poly-L-lysine (Sigma) coated coverslips, or fixed in 1% glutaraldehyde in platelet buffer (145 mM NaCl, 10 mM HEPES [pH 7.4], 10 mM glucose, 0.2 mM Na2HPO4, 5 mM KCl) for 20 min and moved to a coverslip chamber. Images were obtained using 63X differential interference contrast objective equipped with a 1.5X optivar.

Long-Term Transplantation Assay and Analysis
For in vivo HSC analysis, total bone marrow cells (cell doses are indicated in Figure 3) from adult WT and Clasp2 KO male mice were intravenously injected into irradiated (9 Gy split dose, 137Cs-source) female recipients. After 1 and 4 months, donor chimerism (YMT) was analyzed by semiquantitative PCR on peripheral blood. Signal quantitation was by DNA normalization (myogenin) and YMT control DNA dilutions (0, 1, 3, 10, 30, 60 and 100%).

S2  Cell Reports 2, 781–788, October 25, 2012 ©2012 The Authors
In Vivo Homing Assay
Bone marrow (BM) cells were flushed from the tibiae and femura of WT and Clasp2 KO mice with PBS/10% FBS. The mononuclear cells (MNC) were isolated after Ficoll-Hypaque gradient, washed and counted. Cells were then labeled with the cytoplasmic dye carboxyfluorescein diacetate succinimidyl diester (CFSE, Invitrogen) according to manufacturer’s instructions (concentration: 4 μM). More than 99% of the cells were positively stained, with a fluorescence intensity ranging between 10^6 and 10^7 arbitrary units. Part of the CFSE^+ cells was further stained with specific antibodies and analyzed by flow cytometry (BD Biosciences, FACS Aria III) to estimate the percentage of CFSE^+ LSK cells in the cell suspension (this time point was designated 0h). Two doses of CFSE^+ MNC (5 × 10^5 and 12 × 10^5) were intravenously injected into irradiated (900 rads) adult C57Bl/6 recipients. Fifteen hours after injection (15h), the BM cells from each recipient were collected from both femurs and tibias and MNC were isolated after Ficoll. The number of CFSE^+ LSK cells (i.e., from donor origin) that homed into the BM was determined by flow cytometry similar to the 0h time-point. The percentage of WT and Clasp2 KO LSK cells homing into the BM was calculated by dividing the number of donor (CFSE^+) LSK at 15h by the number of CFSE^+ LSK injected at 0h and multiplying this value with 100.

Smarter RNA-Seq-Based Gene Expression Analysis on HSC-Enriched Cells
For RNA sequencing of LSK fractions, cells were pooled from 2 WT mice, and 2 Clasp2 KO mice. For RNA sequencing of CD150^+ LSK, each sample was isolated and converted to cDNA using the Smarter ultra low RNA kit (Clontech). The cDNA was used in the TrueSeq DNA sample prep kit (Illumina v3) to prepare an indexed sequencing library. Samples were sequenced for 36 bp on a HiSeq 2000 using Illumina v3 chemistry.

Illumina BaseCall results were demultiplexed using NARWHAL (Brouwer et al., 2011). The reads were aligned using Tophat (Trapnell et al., 2009) against the UCSC mm9 reference genome, using Ensembl genes.gtf annotation provided by Illumina iGenomes (http://www.illumina.com). For Clasp2 KO LSK mRNA we obtained 45.7 million reads, of which 83.4% aligned back to the reference genome, for WT LSK mRNA we obtained 45.8 million reads with 81.7% alignment, for Clasp2 KO CD150^+ LSK mRNA we obtained 71.7 million reads, with 84.9% alignment, and for WT CD150^+ LSK mRNA we obtained 39.7 million reads with 82.4% alignment. FPKM (Fragments Per Kilobase transcript per Million mapped reads) expression levels were calculated by Cufflinks (Trapnell et al., 2010).

Real-Time PCR
RNA was pooled from 7 WT and 7 Clasp2 KO mice. Flow cytometry was used to isolate the LSK fraction (WT LSK: 0.66 ± 0.23% of live cells, KO LSK: 0.13 ± 0.09 of live cells, p < 0.02 t test). Real time PCR was performed as described (van de Nobelen et al., 2010) to quantify Actin (forward primer: 5’-AGGTCACTCACTATTGGCAAC-3’, reverse primer: 5’-AGAGGTCTTTCAGATGTC-3’) and Meis1 (forward primer: 5’-TCACAGATGGGATAACAG-3’, reverse primer: 5’-ACGGTTTTTGAGCCTT-3’) levels. PCR was performed using Platinum Taq, (Invitrogen, cat no. 10966-034) and the Real-time PCR CFX96 detection system (BIO-RAD).

SUPPLEMENTAL REFERENCES

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Figure S1. Targeted Inactivation of the Clasp2 Gene and Hematopoietic Analyses, Related to Figure 1

(A) Outline of the targeting strategy. The murine Clasp2 gene spans about 200 kb and contains approximately 40 exons (indicated by vertical lines). The second exon common to all known isoforms of CLASP2 was interrupted by insertion of an EGFP-loxP-pMC1NEO-loxP cassette; the GFP sequence is followed by a translational stop, and the neomycin resistance gene (NEO) is transcribed in the opposite direction to Clasp2. LoxP sites (indicated by arrowheads) surround the neomycin resistance gene. The targeting vector contains 1.95 kb of 5' end and 3.1 kb of 3' end homology and a thymidine kinase (TK) gene at the 3' end for counter selection. The targeted Clasp2 allele (Clasp2 KO) is presented at the bottom.

(B) Southern blot analysis. EcoRI-digested ES-cell DNA, from WT mice and heterozygous Clasp2 KO mice (HET), was analyzed with a Clasp2-specific probe. WT and Clasp2 KO bands are indicated. This analysis verifies homologous recombination.

(C) Western blot analyses on brain extracts of WT, heterozygous (HET) and homozygous (HOM) Clasp2 KO mice, using rabbit antibodies against CLASP2 (#2358) and CLASP1 (#2292). The large arrows indicate CLASP2 isoforms, whereas the small arrows indicate CLASP1. CLASP2 is most abundantly expressed in the brain (Akhmanova et al., 2001). Hence, we performed the western blot analysis on this tissue to demonstrate that the targeting of the Clasp2 gene ablated the expression of protein. In WT brain lysates proteins of approximately 140 kDa and 170 kDa were detected, representing the different CLASP2 isoforms (Akhmanova et al., 2001). In extracts of homozygous Clasp2 KO mice these isoforms were not detected, while reduced levels were present in heterozygous mice. A CLASP2 deficiency does not influence CLASP1 expression. CLASP2 is also absent in fibroblasts derived from Clasp2 KO mice (Drabek et al., 2006). We conclude that our targeting strategy successfully abrogates CLASP2 expression.

(D) Western blot analyses on brain extracts of WT, heterozygous (HET) and homozygous (HOM) Clasp2 KO mice, using antibodies against CLIP-115 (#2238, #2221) and CLIP-170 (#2360). Arrows on the left side of the blots indicate CLIP-115, arrows to the right hand side indicate CLIP-170. A CLASP2 deficiency does not influence CLIP expression.

(E) Spleen weight analysis. Whole body and spleen weight of 21 WT mice, and 16 heterozygous (HET) and 22 homozygous Clasp2 KO mice was measured. Both the spleen/body weight ratios (upper panel) as well as the spleen weights (lower panel) are displayed. A t test revealed a significant difference (p < 0.05) between WT and KO, and between HET and KO. SEM is shown.

(F and G) Clonogenic progenitor assays. BFU-E and CFU-E colony assays were performed with bone marrow cells (BM, F) and spleen cells (G). Average and SD are depicted (n > 3 in both groups). t test was applied to calculate significant differences.
Figure S2. Megakaryocyte Analysis, Related to Figure 2
(A) Flow cytometry analysis of bone marrow cells from WT and Clasp2 KO mice aimed at describing megakaryocyte differentiation. 
(B–D) Differential-interference-contrast (DIC) micrographs of proplatelet networks. Megakaryocytes use a MT-based mechanism to convert their cytoplasm into long, branched proplatelets, which remodel into platelets. MT polymerization is necessary to support the enlarging proplatelet mass, which occurs before megakaryocytes shed their platelets (Patel et al., 2005). Although MTs play a crucial role in platelet biogenesis, the MT-based proteins that are involved have not yet been identified. Since CLASP2 is present in proplatelets, as are its interaction partners CLIP-115 and −170 (not shown), EB1 and −3 (Patel et al., 2005), we examined whether CLASP2 deficiency resulted in defects in the formation of proplatelets. Using a megakaryocyte liquid culture system (Italiano et al., 1999) we observed proplatelets, emanating from WT (B) as well as from Clasp2 KO (C, D) megakaryocytes. The CLASP2-deficient proplatelets exhibited the normal beaded morphology observed in WT cultures. Furthermore, ultrastructural analysis of the bone marrow by electron microscopy (EM) showed the presence of mega-karyocytes in Clasp2 KO mice, which contained demarcating membranes and dense granules (data not shown).
(E) Proplatelet production. The percentage of proplatelet-producing megakaryocytes in WT and Clasp2 KO cultures was determined. Megakaryocytes lacking CLASP2 exhibit normal proplatelet production. SD is shown.
(F and G) Immunofluorescence microscopy of resting platelets. Platelets from WT (F), or Clasp2 KO (G) mice were labeled with antibodies to beta-1 tubulin. In several thrombocytopenias associated with mutations in cytoskeletal proteins platelets are large and have an abnormal shape. The normal disc-shape of the platelet is maintained by a marginal band of MTs (Italiano et al., 2003). As CLASP2 is a regulator of the MT network, it might influence the marginal band. We therefore analyzed the shape and cytoskeletal structure of platelets from Clasp2 KO mice. Circulating platelets from Clasp2 KO mice exhibited the normal discoid shape. Furthermore, the diameter and the circumference occupied by MT coils were similar in platelets from WT and Clasp2 KO mice. Magnification is 600x.
(H–M) Immunofluorescence microscopy of activated platelets. Platelets were labeled with phalloidin (green, H, K), which is a marker for F-actin, and with antibodies to beta-1 tubulin (red, I, L). Panels (J) and (M) show merged images. Platelets were from WT (H–J), or Clasp2 KO (K–M) mice. Clasp2 KO platelets, activated with thrombin, spread and formed lamellipodia and filopodia, like WT platelets. Thus, absence of CLASP2 does not significantly impede platelet activation. Magnification is 600x.
Figure S3. A Role for CLASP2 in HSC Maintenance, Related to Figure 3

(A and B) Characterization of HSC-enriched and progenitor cell populations. Representative gating strategy used to sort by flow cytometry LSK (Lin⁻/Sca-1⁺/Kit+) cells, MPPs (myeloid pluripotent progenitors), CMPs (common myeloid progenitors), GMPs (granulocyte-macrophage progenitors), MEPs (megakaryocyte-erythocyte progenitors) in the bone marrow of WT (A) and Clasp2 KO (B) mice. The percentage of LSK+ fractions (i.e., the HSC-enriched cells) in WT and KO mice is shown.

(C) Characterization of cell populations highly enriched for HSCs. Bone marrow cells from 2 WT and 2 Clasp2 KO mice were sorted by flow cytometry as in (A), with the exception that the Lin⁻ cocktail contained CD48, and that CD150 was used in addition to Sca-1 and Kit. The percentage of CD150⁺LSK cells, which is highly enriched for long term HSCs (Kiel et al., 2005) is shown (average values).

(D) Clasp1 and Clasp2 expression in the hematopoietic system. Total RNA was isolated from the LSK+, Lin⁻, and Lin⁺ fractions, as well as from total bone marrow, of WT mice. Real time PCR was performed using Clasp1 and Clasp2 specific primers.

(E) Quantification of Meis1 mRNA. Total RNA was isolated from the LSK fraction of 7 WT mice and 7 Clasp2 KO mice. Expression of Meis1 is shown relative to Actin, as determined by real time PCR (3 experiments, SD indicated, t test reveals significant differences).

(F) Next generation sequencing of LSK-derived mRNA. Total RNA was isolated from the LSK⁻ fraction of 2 WT mice and 2 Clasp2 KO mice. Deep sequencing reveals up- and downregulated genes in KO versus WT (relative expression was calculated with the aid of Cufflinks (right hand columns). The ratio of KO to WT is shown on the left. A list of selected genes is shown (not affected, up-, or downregulated) to confirm that the deep sequencing results are valid, and to show deregulation of interesting genes (see Table S1 for complete analysis). For example, as expected, Clasp2 expression is severely affected in KO samples, whereas the expression of the two genes that are up- and down-stream of the (targeted) Clasp2 locus (Pdcd6ip and Ubp1) is not affected by the insertion of an EGFP-loxP-pMC1NEO-loxP cassette. In addition, the relative expression of Clasp1 (as compared to Actin), determined with deep sequencing (~0.0023 (i.e., ~9 divided by ~4000)), is very similar to that measured by real time PCR (see Figure S3D). The relative expression of Meis1 in WT and KO samples is also very similar in the deep sequencing (Figure S3E) and real time PCR experiments (Figure S3D).
We plated two WT (lines 1 and 8) and two Clasp2 KO (lines 2 and 6) mouse embryonic fibroblast (MEF) cell lines (Drabek et al., 2006) on fibronectin-coated glass coverslips and examined the appearance of focal adhesions and stable MTs 90 min after plating using immunofluorescence analysis. Upon adherence to fibronectin, both WT cells and Clasp2 KO cells formed focal adhesions, as detected with an antibody against phosphorylated focal adhesion kinase (FAK-pY397, data not shown). Neither of the two Clasp2 knock out MEF lines (B, D, F) form stable MTs as efficiently as the WT lines (A, C, E), as measured with two different markers for stable MTs (Bulinski and Gundersen, 1991), i.e., anti-acetylated (acet) tubulin and anti-detyrosinated (glu) tubulin antibodies. Moreover, Clasp2 KO MEFs adhered less efficiently to a fibronectin-coated surface than WT cells (data not shown). Clasp2 knock out MEFs did form a normal MT array (G, H) and EB3 was localized at the ends of MTs in Clasp2 knock out fibroblasts (I, J). As a further control we tested adult dermal fibroblast lines instead of MEFs, and obtained a similar result, i.e., CLASP2 is required for the formation of stable MTs upon cell attachment (data not shown). Furthermore, we were able to rescue the MT stabilization defect in Clasp2 KO MEFs by expressing GFP-CLASP2 (data not shown).