ARTICLE

Cdk6 contributes to cytoskeletal stability in erythroid cells

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ABSTRACT

Mice lacking Cdk6 kinase activity suffer from mild anemia accompanied by elevated numbers of Ter119$^+$ cells in the bone marrow. The animals show hardly any alterations in erythroid development, indicating that Cdk6 is not required for proliferation and maturation of erythroid cells. There is also no difference in stress erythropoiesis following hemolysis *in vivo*. However, *Cdk6*$^{-/-}$ erythrocytes have a shortened lifespan and are more sensitive to mechanical stress *in vitro*, suggesting differences in cytoskeletal architecture. Erythroblasts contain both Cdk4 and Cdk6, while mature erythrocytes apparently lack Cdk4 and their Cdk6 is partly associated with the cytoskeleton. We used mass spectrometry to show that Cdk6 interacts with a number of proteins involved in cytoskeleton organization. *Cdk6*$^{-/-}$ erythroblasts show impaired F-actin formation and lower levels of gelsolin, which interacts with Cdk6. We also found that Cdk6 regulates the transcription of a panel of genes involved in actin (de-)polymerization. *Cdk6*-deficient cells are sensitive to drugs that interfere with the cytoskeleton, suggesting that our findings are relevant to the treatment of patients with anemia – and may be relevant to cancer patients treated with the new generation of CDK6 inhibitors.

Introduction

Cyclins and cyclin-dependent kinases (Cdk) are critical regulators of the cell cycle. Cyclin/Cdk complexes trigger cell-cycle progression with D-type cyclins and Cdk responsible for controlling the early G_1 phase of the cell cycle. In the early G_1 phase, cyclin-D-Cdk4/6 complexes phosphorylate and inactivate the retinoblastoma protein to activate E2F-dependent transcription.[1] Cdk4 and Cdk6 are 71% identical at the amino acid level, are ubiquitously expressed and bind all three D-type cyclins.[2] Only the combined deletion of *Cdk4* and *Cdk6* induces late embryonic lethality due to defects in hematopoiesis,[3,4] while deletion of either gene alone is not fatal. Accordingly, Cdk4 and Cdk6 are considered to have redundant functions in regulating cell-cycle progression.

Nevertheless, the lack of either of the kinases leads to specific defects in particular types of cells. *Cdk4*$^{-/-}$ mice have defective postnatal pancreatic beta cells and pituitary cells,[5,6] while the lack of Cdk6 leads to disorders in the hematopoietic compartment such as altered thymocyte development and lower numbers of red blood cells and cells of other hematopoietic lineages.[4,7,8] Recent evidence indicates that Cdk6 is a key player in the differentiation of a variety of cell types, a function not shared by Cdk4. A decline in the level of Cdk6 is required for terminal differentiation of

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murine erythroid leukemia cells[9] and Cdk6 expression in astrocytes has been associated with the expression of progenitor cell markers.[10] Osteoblast differentiation has been described to be inhibited by overexpression of Cdk6 but not of Cdk4.[11] A related study showed that Cdk6 protein levels are downregulated by Rankl-induced osteoclast differentiation.[12] Moreover, Cdk6 inhibits the transcriptional activation of Runx1 and thus blocks myeloid differentiation.[13] Cdk6 kinase activity is also required for thymocyte development, in which it functions by modulating the expression of Notch target genes.[8] Cdk6 but not Cdk4 regulates transcription of key players in lymphoma formation and myeloid leukemia in a kinase-dependent and/or kinase-independent manner.[14–16] Cdk6 has also been ascribed a unique role in actin dynamics in astrocytes:[10,17] overexpression of Cdk6 in primary mouse astrocytes results in altered gene expression and modified cytoskeletal architecture, as well as in loss of stress fibers and enhanced motility.

Erythrocytes transport oxygen through the body. They have a unique biconcave shape that allows a certain flexibility when travelling through narrow capillary vessels.[18] Changes of form are made possible by the delicate interplay between the fluid cell membrane and the structure of the membrane cytoskeleton.[18] Mutations in genes encoding actin-binding proteins such as adducin, dematin, tropomyosin, tropomodulin and protein 4.1 are known to cause altered actin stability and increased erythrocyte fragility leading to anemia. The correct polymerization and organization of actin is vital for the strength and flexibility of erythrocytes.[18] Besides, actin-remodeling proteins are required for regulating actin filament length. Among those, gelsolin, a Ca^{++}-dependent protein, severs preassembled actin filaments and is hence crucial in actin remodeling.[19]

We show that the anemic phenotype of mice lacking Cdk6 kinase activity is not attributable to a reduced production of erythrocytes but is the consequence of a reduced erythroid life span. Our findings reveal a novel role for Cdk6 as a stabilizer of the erythrocyte cytoskeleton via the deregulation of genes involved in cytoskeleton stability.[20–22]

Methods

Mouse strains

Mice were maintained under pathogen-free conditions at the Institute of Pharmacology and Toxicology, University of Veterinary Medicine, Vienna (Austria). C57BL/6 mice are referred to as *Cdk6*$^{+/+}$. *Cdk6*$^{-/-}$ [4] and *Cdk6*$^{K43M/K43M}$ [8] mice were generated on the C57BL/6 background. Mice aged 6-8 weeks were used unless otherwise indicated. Blood parameters were analyzed with a VetABC blood counter.

Flow cytometry

Bone marrow, spleen and fetal liver cells were stained for erythroid subsets using the antibodies listed below. Samples were analyzed by a FACSCantoII flow cytometer using FACSDiva software (Becton-Dickinson). Erythrocyte development in the bone marrow and spleen was analyzed as follows: Ter119^{med}CD71^{high} (proerythroblasts; R1), Ter119^{high}CD71^{high} (basophilic erythroblasts; R2), Ter119^{high}CD71^{med} (late basophilic and polychromatophilic erythroblasts; R3) and Ter119^{high}CD71^{low} (orthochromatophilic erythroblasts; R4). Cells were stained with the fluorescently labeled antibodies: Ter119-PE (eBioscience), CD71 Biotin (eBioscience), Streptavidin eflour 780 (eBioscience), CD49e PE (integrin α5), and CD49d FITC (integrin α4).

In vivo biotin labeling

The life span of red blood cells *in vivo* was measured by injecting 3mg EZ-Link-sulfo-NHS-biotin (Pierce, Rockford, IL, USA) into mice. Decay of the labeled red blood cells was measured by FACS analysis.[23]

Osmotic fragility

Fresh blood from age-matched mice was washed twice in phosphate-buffered saline. Osmotic fragility was measured by mixing 25 µL blood with 2.5 mL saline solutions (with salt concentrations between 0 and 0.9% NaCl). After gentle mixing the suspension was incubated for 15 min at room temperature and centrifuged at 500 x g for 10 min. The osmotic fragility curve was obtained by plotting the measured absorbance of hemoglobin released into the supernatants at 540 nm for each solution against NaCl concentrations. Duplicates for each NaCl concentration point were read.

Measurement of membrane mechanical stability

Fresh murine blood was taken, diluted in 10 volumes of phosphate-buffered saline and subjected to a constant shear stress of 29G, 0.33 x 12 mm. The suspension was centrifuged at 500 x g for 10 min. The absorbance of hemoglobin supernatant was measured at 540 nm. Fresh human erythrocytes were diluted in 10 volumes of phosphate-buffered saline and incubated with palbociclib (manufactured by Pfizer) at indicated concentrations overnight at 37°C, in 5% CO_2 and 95% humidity. Dimethyl sulfoxide (DMSO) was used as the vehicle control. The degree of resistance of red blood cells to mechanical stress was measured as above.

Study approval

Venous blood was drawn from healthy volunteers after informed consent. Animal experiments were performed in accordance with protocols approved by Austrian law and the Animal Welfare Committee at the University of Veterinary Medicine, Vienna.

Statistical analysis

Statistical analysis was carried out using a two-tailed unpaired Student *t* test and one-way or two-way analysis of variance (ANOVA) as appropriate. Data are presented as mean values ± standard error of the mean (SEM) and were processed using GraphPad software.

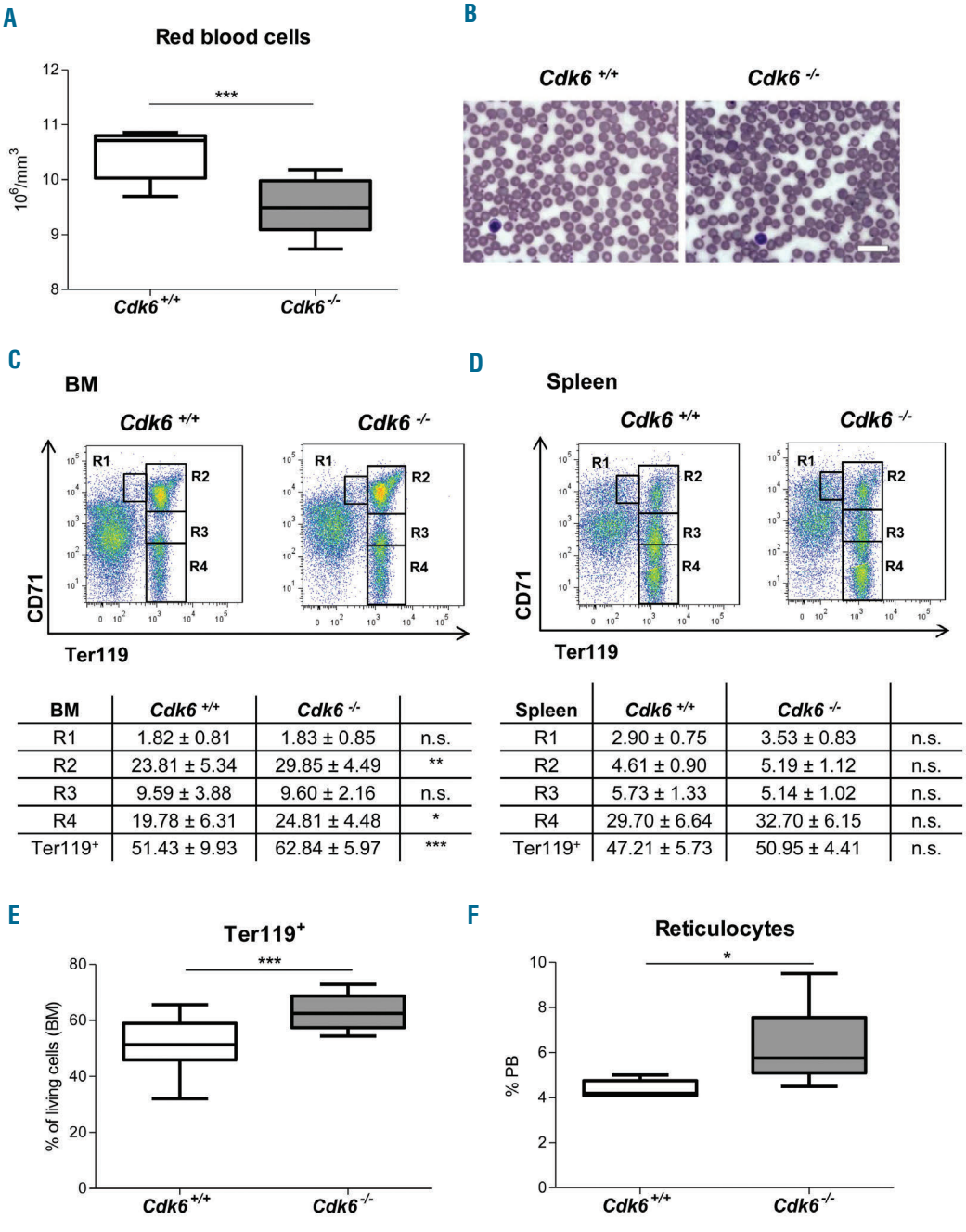
Results

Cdk6$^{-/-}$ mice have anemia accompanied by an increased number of Ter119^{+} cells in the bone marrow

Cdk6$^{-/-}$ mice are viable and fertile and display minor defects in hematopoiesis with anemia and reduced cellularity in thymus and spleen.[4] The reduction in erythrocyte numbers is paralleled by an increase in mean cell volume and mean corpuscular hemoglobin.[4] We confirmed these observations in C57BL/6 *Cdk6*$^{-/-}$ mice under our housing conditions (Figure 1A,B and *Online Supplementary Figure S1A*).

Anemia may be caused by enhanced hemolysis or altered iron metabolism. We analyzed levels of plasma iron, plasma hepcidin (a key iron regulator) and erythropoietin (a major mitogen for erythroid progenitors). No

significant changes were detected although there was a tendency towards higher plasma iron and erythropoietin levels in *Cdk6*$^{-/-}$ mice (*Online Supplementary Figure S1B-D*). Likewise, the ability to form myeloid colonies was not altered among genotypes (*Online Supplementary Figure S1E*). Analysis of the maturation of erythroid progenitors in the bone marrow and spleen by FACS revealed minor differences: a profiling developed by Socolovsky *et al.*,[24] showed slightly enhanced numbers of cells in the R2 (basophilic erythroblasts) and R4 (orthochromatophilic erythroblasts) fractions in the bone marrow but not in the spleen (Figure 1C,D). The increased amount of erythro-



BM	*Cdk6* $^{+/+}$	*Cdk6* $^{-/-}$	
R1	1.82 ± 0.81	1.83 ± 0.85	n.s.
R2	23.81 ± 5.34	29.85 ± 4.49	**
R3	9.59 ± 3.88	9.60 ± 2.16	n.s.
R4	19.78 ± 6.31	24.81 ± 4.48	*
Ter119$^+$	51.43 ± 9.93	62.84 ± 5.97	***

Spleen	*Cdk6* $^{+/+}$	*Cdk6* $^{-/-}$	
R1	2.90 ± 0.75	3.53 ± 0.83	n.s.
R2	4.61 ± 0.90	5.19 ± 1.12	n.s.
R3	5.73 ± 1.33	5.14 ± 1.02	n.s.
R4	29.70 ± 6.64	32.70 ± 6.15	n.s.
Ter119$^+$	47.21 ± 5.73	50.95 ± 4.41	n.s.

Figure 1. *Cdk6*$^{-/-}$ mice are anemic with compensatory upregulation of erythrocytes in the bone marrow. (A) *Cdk6*$^{-/-}$ mice have a reduced red blood cell count (n of *Cdk6*$^{+/+}$ mice = 7; n of *Cdk6*$^{-/-}$ mice = 11). A two-tailed unpaired Student *t* test was used for the statistical analysis. Error bars indicate ± SEM (***P<0,001). (B) One representative Giemsa staining of *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ blood is depicted (scale bar 20 μm; microscope: Zeiss AxioImager Z2; lens: Zeiss plan-apochromat 63x/1.4 oil lens; acquisition software: ZEN 2012). (C-D) FACS staining of erythroid development in the bone marrow (BM) and spleen of *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ mice determined by the surface marker CD71/Ter119: Ter119^{med}CD71^{high} (proerythroblasts), Ter119^{high}CD71^{high} (basophilic erythroblasts), Ter119^{high}CD71^{med} (late basophilic and polychromatophilic erythroblasts) and Ter119^{high}CD71^{low} (orthochromatophilic erythroblasts) in regions R1-R4, respectively. The distribution of the respective populations is summarized in the lower panels. Statistical analysis was performed with a two-tailed unpaired Student *t* test in bone marrow (n=17 per genotype) and spleen (n≥7 per genotype); n.s.: not significant; *P<0.05; **P<0.01; ***P<0.001). (E) *Cdk6*$^{-/-}$ mice have increased total numbers of Ter119$^+$ erythrocytes in the bone marrow (BM) (n=6 per genotype). A two-tailed unpaired Student *t* test was used for the statistical comparison. Error bars indicate ±SEM (***P<0.001). (F) Reticulocyte numbers in the peripheral blood (PB) were analyzed by FACS using thiazole orange (n=5 per genotype). Reticulocyte counts were increased in *Cdk6*$^{-/-}$ mice, balancing the reduced red blood cell count. Statistical analysis was carried out using a two-tailed unpaired Student *t* test. Error bars indicate ± SEM (*P<0.05).

cyte precursors became most evident from the highly significant increase in total Ter119+ erythroid cells in the bone marrow of *Cdk6*$^{-/-}$ animals (Figure 1E). No differences were detected in the spleen (*Online Supplementary Figure S2A*) or during erythrocyte maturation in fetal liver erythropoiesis (at day E13) between *Cdk6*$^{-/-}$ and wildtype controls (*Online Supplementary Figure S2B*).

One explanation for the increased proportion of Ter119+ cells in the bone marrow accompanying anemia in the peripheral blood is a reduced ability of mature red blood cells to exit the bone marrow. To enter the periphery, red cells downregulate surface integrins during erythropoiesis. FACS staining revealed no differences in integrin patterns: levels of downregulation of integrin α4 (*Online Supplementary Figure S2C*) and integrin α5 (*Online Supplementary Figure S2D*) were comparable irrespective of the genotype. The unaltered ability of *Cdk6*$^{-/-}$ erythrocytes to migrate efficiently from the bone marrow to the periphery was also visible from the elevated numbers of reticulocytes (which contain residual amounts of RNA that can be stained with thiazole orange) in the blood. *Cdk6*$^{-/-}$ mice had significantly more reticulocytes in the peripheral blood than had *Cdk6*$^{+/+}$ controls (Figure 1F) reflecting the enhanced production of red blood cells. In summary, a reduced erythroid development in the bone marrow does not account for anemia in *Cdk6*$^{-/-}$ mice.

Cdk6$^{-/-}$ mice respond normally to erythropoietic stress

The bone marrow reacts immediately to conditions of high need by increasing the production of erythrocytes through a process referred to as stress erythropoiesis. We reasoned that subtle changes in erythropoiesis may become more pronounced under conditions of stress. Two independent experimental settings were used to investigate whether *Cdk6*$^{-/-}$ mice respond to hypoxia by increasing erythroid production. First, we mimicked stress erythropoiesis *in vitro*: fetal liver erythroblasts (day E13) can be cultured and stimulated to proliferate and differentiate by use of distinct cytokines.[25] *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ erythroblasts displayed superimposable growth curves (Figure 2A) and similar morphology (Figure 2B). A proliferation assay using CellTrace Violet showed no differences among genotypes (*Online Supplementary Figure S3A*). Differentiating erythrocytes divide approximately four times before they mature to fully differentiated enucleated red cells.[25] The kinetics of differentiation of *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ erythroblasts were comparable, as shown by growth curves (Figure 2C), the Violet Cell proliferation assay (*Online Supplementary Figure S3B*) and benzidine staining (*Online Supplementary Figure S3C*).

The *in vitro* findings were echoed by *in vivo* studies: mice were exposed to phenylhydrazine which induces hemolytic anemia and forces the subsequent rapid expansion of the erythroid lineage in the bone marrow and spleen.[26,27] *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ mice showed a comparable recovery response when analyzed 10 days after receipt of a single dose of phenylhydrazine. Red blood cell count (Figure 2D) and hematocrit (*Online Supplementary Figure S3D*) were restored to normal levels. Massive increases in numbers of all premature stages (R1-R2) of the erythroid lineage were detectable in the bone marrow (*Online Supplementary Figure S3E*) and the spleen (*Online Supplementary Figure S3F*) 3 days after phenylhydrazine induction irrespective of the genotype. We only observed one subtle difference: whereas the proportion of Ter119+ erythroid cells in the wildtype bone marrow increased from ~50% to ~60%, it remained consistently high at 60% in *Cdk6*$^{-/-}$ bone marrow (Figure 2E). This difference was not seen in the spleen: numbers of Ter119+ cells increased to the same extent in *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ mice upon phenylhydrazine treatment (Figure 2F).

Cdk6$^{-/-}$ erythrocytes have a decreased life span *in vivo*

We next analyzed the possibility that the anemia in *Cdk6*$^{-/-}$ mice is linked to a shortened lifespan of the cells. Peripheral erythrocytes were labeled *in vivo* with EZ-Link sulfo-NHS-biotin and blood samples were taken every 10 days. In line with published data,[28,29] we found that under these conditions wildtype erythrocytes had an average half-life of 19 days, while that of erythrocytes from *Cdk6*$^{-/-}$ mice was significantly shorter (14 days; Figure 3A). The shortened half-life may be indicative of a lower mechanical stability of the cells as the long-term survival of mature erythrocytes in the periphery demands mechanical strength and flexibility. Freeze-thaw cycles also pose a challenge to the mechanical stability of cells. Notably, we found that erythroblasts from *Cdk6*$^{-/-}$ fetal livers did not survive a freeze-thaw cycle (Figure 3B). Changes in membrane stability may also be associated with reduced tolerance to osmotic stress which was tested using various concentrations of NaCl. However, osmotic sensitivity was not altered between *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ erythrocytes (Figure 3C).

Cdk6 is tethered to the cell membrane in erythrocytes

We next tested whether the absence of Cdk6 is associated with changes of the cytoskeleton. The major cytoskeletal components (spectrin, ankyrin, band 3 and protein 4.1/4.2) were present at comparable levels irrespective of the genotype (*Online Supplementary Figure S4*). However, phalloidin staining of fixed erythroblasts revealed a pronounced reduction in filamentous-actin (F-actin) polymerization upon loss of Cdk6 (Figure 4A and *Online Supplementary Figure S5A,B*) although the level of total beta-actin (B-actin, non-polymerized actin) in erythroblasts was unaffected (Figure 4B). In mature erythrocytes differences in phalloidin staining were still observed, albeit to a lesser extent: in wildtype erythrocyte cytoskeleton F-actin structures were symmetrical and had a pronounced ring shape whereas the actin oligomers in *Cdk6*$^{-/-}$ erythrocytes were distributed diffusely (*Online Supplementary Figure S5C*). In line, *Cdk6*-deficient lymphoid cells had a decreased filamentous to globular actin ratio suggesting the interplay between Cdk6 and actin structures is not restricted to erythroid cells (*Online Supplementary Figure S5D*).

Proliferating erythroblasts express both cell-cycle kinases Cdk4 and Cdk6 (Figure 4B). Mature erythrocytes still contain Cdk6 protein (Figure 4C) whereas Cdk4 is degraded during cell maturation. Cdk4 was only present at low levels in *Cdk6*$^{-/-}$ erythrocytes (Figure 4C). Erythrocyte membranes (ghosts) contain mainly cytoskeletal and membrane proteins but also some peripheral and membrane-associated proteins. It is therefore of particular interest that we detected significant amounts of Cdk6 but not Cdk4 in ghosts (Figure 4D and *Online Supplementary Figure S5E*). These data indicate that Cdk6 is maintained in mature erythrocytes; a proportion is associated with the membrane and may play an active role in regulating cytoskeletal proteins. These findings are in line with a model that Cdk6 is tethered to the cytoskeleton where it may be protected from degradation and have a membrane stabilizing role.[17]

Cdk6 exerts kinase-dependent effects on erythrocyte stability

Cdk6 may affect cytoskeleton and membrane stability in a kinase-dependent and/or kinase-independent manner. Mice harboring a kinase dead mutant of Cdk6 (*Cdk6*$^{K43M/K43M}$)[8] recapitulate the phenotype of *Cdk6*$^{-/-}$ animals: we found decreased red blood cell counts accompanied by an increase in mean cell volume (Figure 5A and *Online Supplementary Figure S6A*). No gross morphological alterations were detected (Figure 5B). Reminiscent of *Cdk6*$^{-/-}$ mice we found alterations in the stages of erythroid development in the bone marrow and an increased total

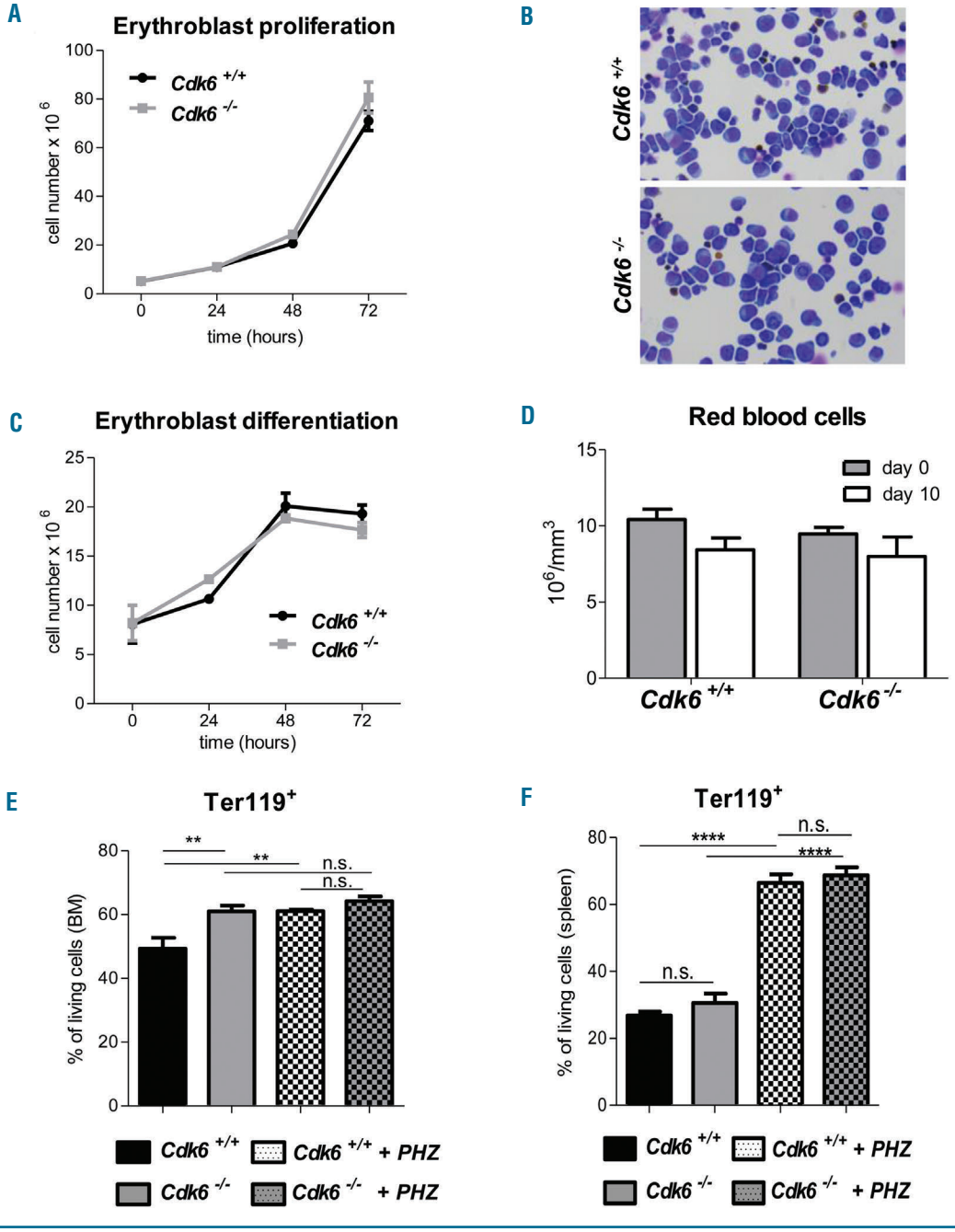


Figure 2. Stress erythropoiesis is unaffected upon loss of *Cdk6*. (A) Proliferation of *in vitro* cultured *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ erythroblasts is comparable (n=3 per genotype). Error bars indicate ± SEM. (B) Immunohistochemical staining of erythroblasts using neutral benzidine and histological dyes. (C) Differentiation was induced by a change in growth factors added to the medium. Erythroblasts divide a few times before enucleation and contraction starts. Cell numbers are shown. Error bars indicate ± SEM (n=3 per genotype). (D-F) *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ animals were treated twice with phenylhydrazine (PHZ) to induce hemolytic anemia. (D) *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ mice are able to restore red blood cell counts to normal levels to a comparable extent on day 10 after PHZ challenge. Error bars indicate ± SEM (n≥4 per genotype). Two-way ANOVA was used for the statistical comparison. (E) The total amount of Ter119^{+} erythrocytes in the bone marrow (BM) is already enriched in untreated Cdk6-deficient bone marrow with no further increase upon phenylhydrazine (PHZ) treatment. A two-tailed unpaired Student *t* test was used for the statistical comparison. Error bars indicate ± SEM (n≥4 per genotype; $^{**}P<0.01$; n.s.: not significant). (F) Phenylhydrazine (PHZ) treatment results in elevated total amount of Ter119^{+} erythrocytes in spleen independent of the genotype. Statistical analysis was performed with a two-tailed unpaired Student *t* test. Error bars indicate ± SEM (n≥4 per genotype; $^{****}P<0.0001$ n.s.: not significant).

number of Ter119^{+} erythroid cells (Figure 5C,D). Analysis of erythroid progenitors in the spleen and numbers of reticulocytes in the peripheral blood were not significantly altered (*Online Supplementary Figure S6B,C*). However, as seen in *Cdk6*-deficiency, *Cdk6*$^{K43M/K43M}$ erythroid cells showed a decreased filamentous to globular actin ratio (Figure 5E). These data suggest a kinase-dependent function of Cdk6 in cytoskeletal integrity.

Cdk6 transcriptionally regulates genes involved in cytoskeleton organization in a kinase-dependent manner

To investigate which cytoskeleton components are altered upon loss of Cdk6 kinase activity, we performed quantitative polymerase chain reaction studies of a set of genes that had been implicated in cytoskeleton remodeling in the literature using erythroid progenitors with intact nuclei [Ter119^{high}CD71^{low} (orthochromatophilic erythroblasts; R4)] (Figure 6A and *Online Supplementary Figure S7A*). In the absence of Cdk6 kinase activity we found significant changes in the mRNA levels of four genes: *Gelsolin*, *Tubulin alpha-8*, *Baiap2* and *Pip5k1b* were reduced to ~50% (Figure 6A). Cdk6 directly regulates the transcription of *Gelsolin* and *Baiap2* as we were able to further show enhanced binding of Cdk6 to the respective promoters by chromatin immunoprecipitation assays in the non-transformed mouse progenitor HPC7 cell line and in *Bcr/Abl*$^{p185+}$-transformed lymphoid cells (Figure 6B). Lymphoid cell expression of gelsolin, a known regulator of actin filament (de-)polymerization,[19] was also decreased in *Cdk6*$^{K43M/K43M}$ mice (*Online Supplementary Figure S7B*).

Intrigued by the observation that Cdk6 regulates the expression of genes involved in cytoskeleton organization in all our model systems we investigated which components of the cytoskeleton or cell membrane are bound to Cdk6. We immunoprecipitated Cdk6 from wildtype peripheral blood followed by mass spectrometry and complemented the analysis by including Cdk6 immunoprecipitation from wildtype lymphoid cells. The detection of Cdk6 protein served as a positive control. Mass spectrometry verified a set of potential Cdk6 interactors (*Online Supplementary Figure S8*). In mature erythrocytes we confirmed the interaction with gelsolin and also detected a set of proteins that have been associated with anemia and/or erythroid cytoskeletal organization/dynamics (Figure 6C and *Online Supplementary Figure S8*). Tubulin was an attractive candidate in lymphoid cells (*Online Supplementary Figure S8*).

Cdk6$^{-/-}$ cells display an increased sensitivity to shear stress and drugs interfering with cytoskeleton organization

On this basis, we also reasoned that *Cdk6*$^{-/-}$ cells should be more susceptible to any pharmacological disturbance of the cytoskeleton. We perturbed either actin metabolism

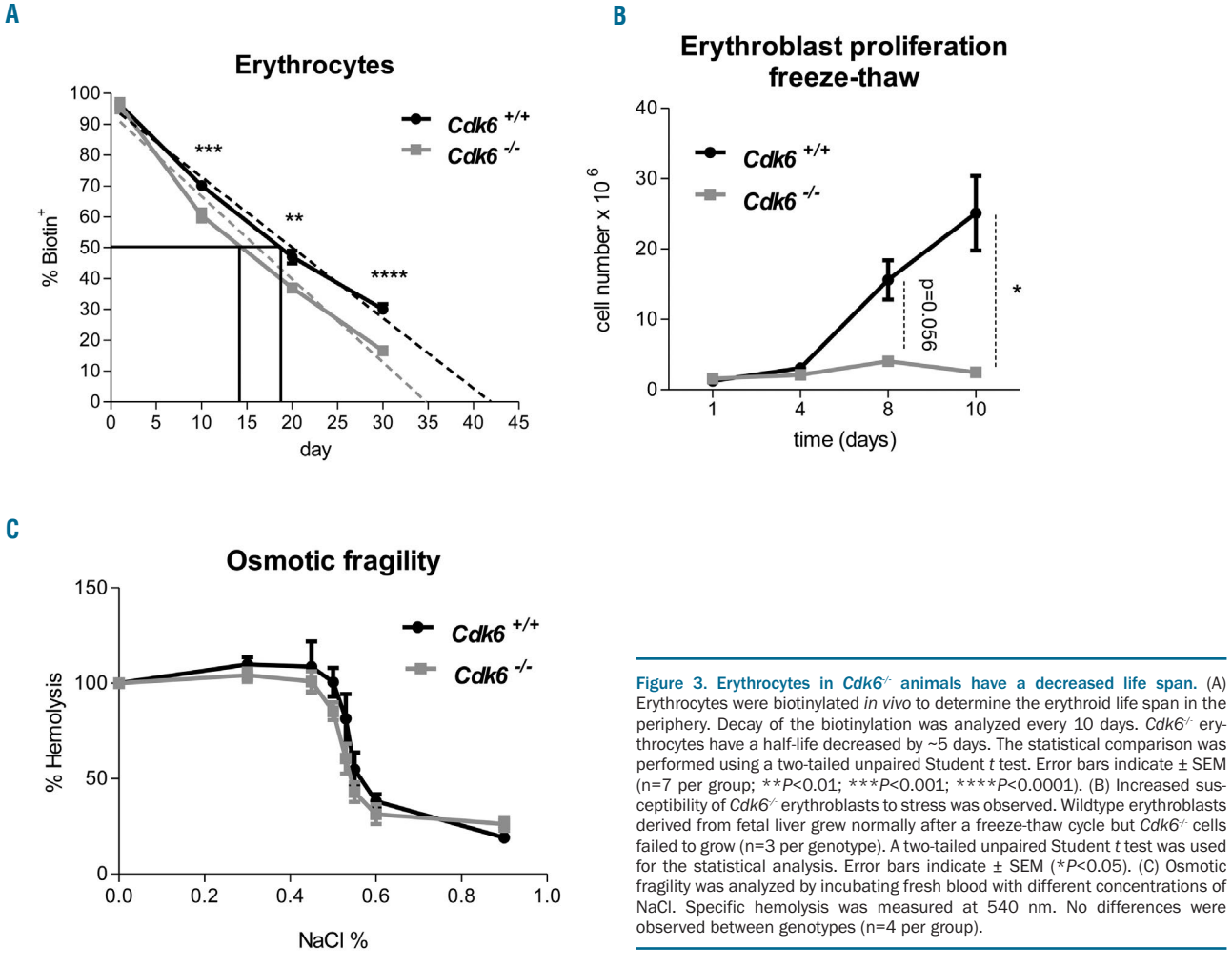


Figure 3. Erythrocytes in *Cdk6*$^{-/-}$ animals have a decreased life span. (A) Erythrocytes were biotinylated *in vivo* to determine the erythroid life span in the periphery. Decay of the biotinylation was analyzed every 10 days. *Cdk6*$^{-/-}$ erythrocytes have a half-life decreased by ~5 days. The statistical comparison was performed using a two-tailed unpaired Student *t* test. Error bars indicate ± SEM (n=7 per group; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$). (B) Increased susceptibility of *Cdk6*$^{-/-}$ erythroblasts to stress was observed. Wildtype erythroblasts derived from fetal liver grew normally after a freeze-thaw cycle but *Cdk6*$^{-/-}$ cells failed to grow (n=3 per genotype). A two-tailed unpaired Student *t* test was used for the statistical analysis. Error bars indicate ± SEM ($^{*}P<0.05$). (C) Osmotic fragility was analyzed by incubating fresh blood with different concentrations of NaCl. Specific hemolysis was measured at 540 nm. No differences were observed between genotypes (n=4 per group).

by the addition of jasplakinolide,[19] which stabilizes actin filaments, or microtubule metabolism by the addition of two benzimidazoles (albendazole and thiabendazole),[30] which block tubulin polymerization. The treatments showed that our prediction was respected: *Cdk6*$^{-/-}$ cells were significantly more sensitive (Figure 6D). Combined treatment with CDK6 kinase inhibitor and microtubule poison mimicked the absence of *Cdk6* in wildtype cells: the Bliss additivity model revealed an *in vitro* synergistic cytotoxicity between palbociclib and albendazole (*Online Supplementary Figure S9A*).

Fragility of erythrocytes can be tested by exposing cells to shear stress: *Cdk6*$^{-/-}$ and *Cdk6*$^{K43M/K43M}$ erythrocytes reacted with an enhanced lysis (Figure 6E). Similar observations although not reaching the level of statistical significance were made upon CDK4/6 kinase inhibitor treatment in human erythroid cells (*Online Supplementary Figure S9B*). Collectively, these data indicate a global role for Cdk6 as a regulator of membrane and cytoskeleton stability which manifests itself as anemia in the red cell compartment.

Discussion

Anemia is a frequent disorder with a variety of possible causes. It most frequently stems from reduced erythrocyte formation due to a lack of iron, vitamin B12 or folic acid[31–33] but may also be associated with increased or premature destruction of erythrocytes in the periphery.[34] Anemia of this latter type is generally caused by alterations in the cytoskeleton and the membrane and a large number of erythrocyte disorders are associated with mutations in

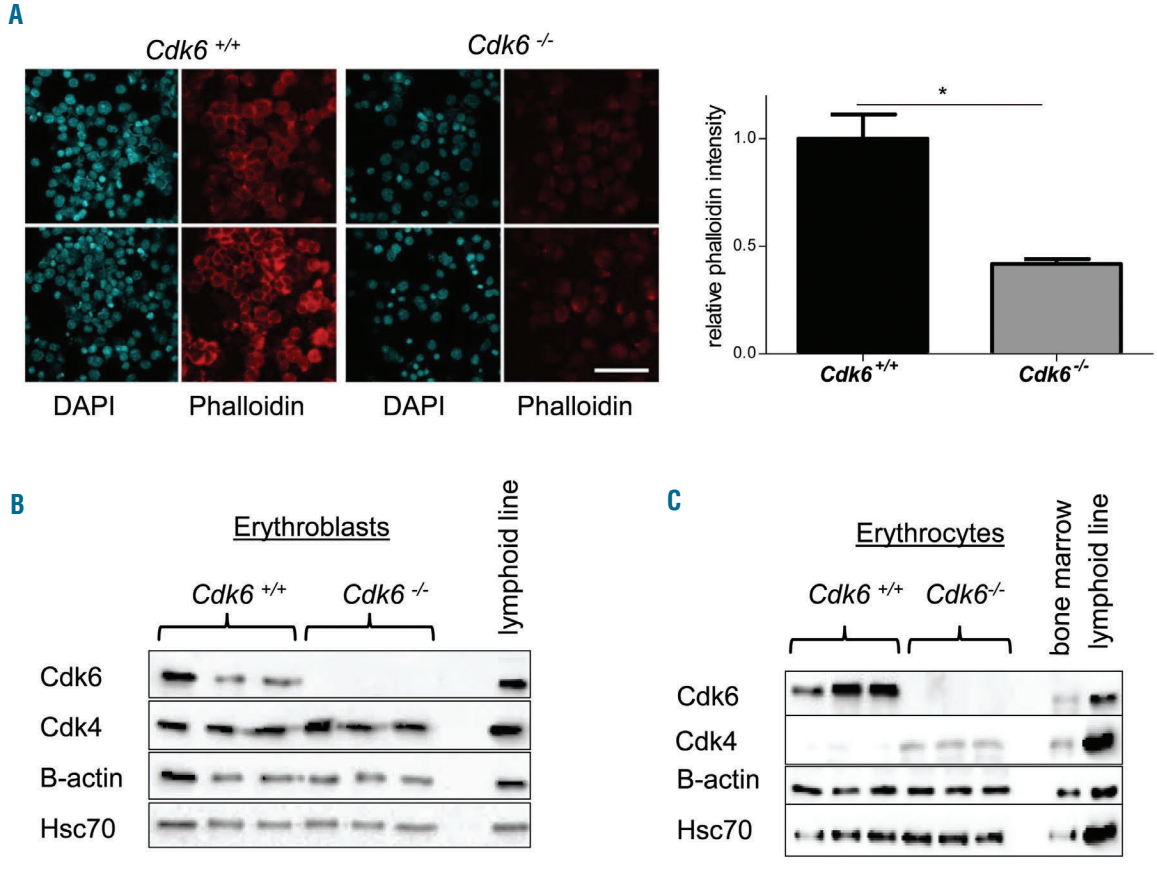
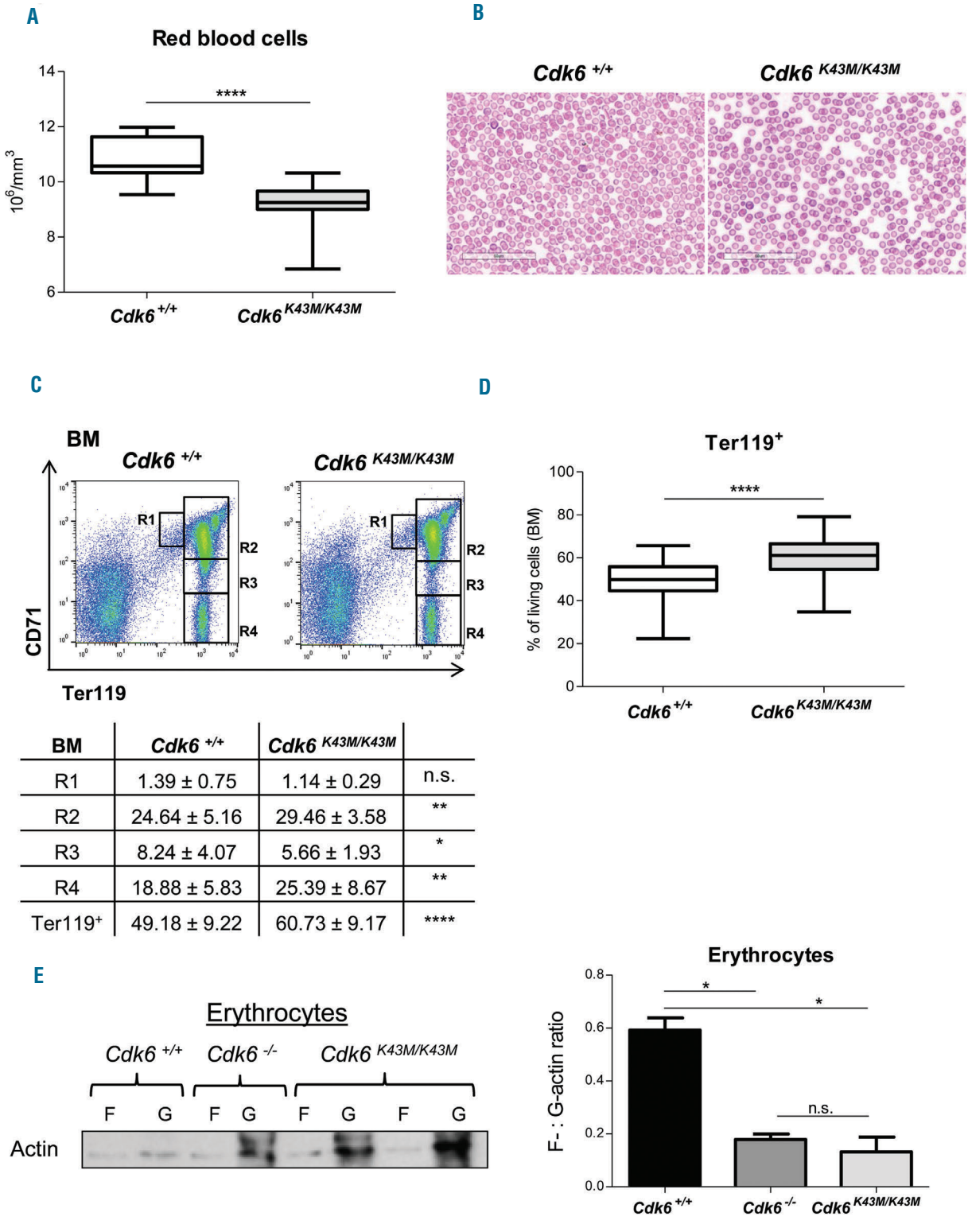


Figure 4. Altered cytoskeletal composition in *Cdk6*$^{-/-}$ erythroid cells. (A) Phalloidin immunofluorescence staining revealed decreased amounts of filamentous (F-) actin in *Cdk6*$^{-/-}$ erythroblasts (scale bar 50 μm; microscope: Zeiss LSM 510 Meta; Axiovert 200M; lens: Zeiss plan-apochromat 63x/1.4 oil lens; fluorochromes: DAPI, Alexa 546 (coupled phalloidin); acquisition software: ZEN 2009). The right panel depicts the summary of three experiments. Statistical analysis was conducted by a two-tailed unpaired Student *t* test. Error bars indicate ± SEM (*P<0.5). (B) Proliferating erythroblasts exhibited normal expression levels of Cdk4 and Cdk6 and no differences in beta-actin (in its non-polymerized form) as quantified by western blots. Three biological replicates per genotype are shown. Anti-Hsc70 antibody was used as loading control. A lymphoid cell line served as positive control. (C) Mature erythrocytes showed elevated levels of Cdk6. Only a weak Cdk4 signal was detectable in *Cdk6*$^{-/-}$ erythrocytes by immunoblotting. Actin levels were similar. Three biological replicates per genotype are depicted. Whole bone marrow and a lymphoid cell line were used as positive controls. Anti-Hsc70 antibody served as loading control. (D) In erythrocyte membranes (ghosts) Cdk6 but not Cdk4 protein was detectable. Actin levels remained comparable. Anti-Hsc70 antibody was used as loading control. Three biological replicates per genotype are shown. Whole bone marrow and a lymphoid cell line served as positive controls.



BM	*Cdk6* $^{+/+}$	*Cdk6* $^{K43M/K43M}$	
R1	1.39 ± 0.75	1.14 ± 0.29	n.s.
R2	24.64 ± 5.16	29.46 ± 3.58	**
R3	8.24 ± 4.07	5.66 ± 1.93	*
R4	18.88 ± 5.83	25.39 ± 8.67	**
Ter119$^+$	49.18 ± 9.22	60.73 ± 9.17	****

Figure 5. *Cdk6*$^{K43M/K43M}$ mice show mild anemia with compensatory upregulation of erythrocytes in the bone marrow. (A) *Cdk6*$^{K43M/K43M}$ mice have a reduced red blood cell count (n of *Cdk6*$^{+/+}$ mice=19; n of *Cdk6*$^{K43M/K43M}$ mice=17). Statistical analysis was carried out using a two-tailed unpaired Student *t* test. Error bars indicate ± SEM (****P<0.0001). (B) One representative Giemsa staining of *Cdk6*$^{K43M/K43M}$ and *Cdk6*$^{+/+}$ blood is depicted (scale bar 60 μm). (C) FACS staining of the erythroid development in the bone marrow (BM) was determined by the surface marker CD71/Ter119 as described in Figure 1C,D (n≥16 per genotype). A two-tailed unpaired Student *t* test was used for the statistical analysis. Error bars indicate ± SEM (n.s.: not significant; *P<0.05; **P<0.01; ****P<0.0001). R1: Ter119^{med}CD71^{high} (proerythroblasts); R2: Ter119^{high}CD71^{high} (basophilic erythroblasts); R3: Ter119^{high}CD71^{med} (late basophilic and polychromatophilic erythroblasts) and R4: Ter119^{high}CD71^{low} (orthochromatophilic erythroblasts). (D) *Cdk6*$^{K43M/K43M}$ mice had increased total numbers of Ter119$^+$ erythrocytes in the bone marrow (BM) (n of *Cdk6*$^{+/+}$=36; n of *Cdk6*$^{K43M/K43M}$=21). Statistical comparison was conducted by a two-tailed unpaired Student *t* test. Error bars indicate ± SEM (****P<0.0001). (E) One representative blot shows the amount of F-actin content *versus* G-actin content in mature red blood cells of indicated genotypes. The right panel depicts the summary of three experiments. A two-tailed unpaired Student *t* test was used for the statistical analysis. Error bars indicate ± SEM (*P<0.05; n.s.: not significant). F: filamentous F-actin; G: free globular G-actin.

genes required for membrane and cytoskeleton stability.[18] Such mutations render erythrocytes susceptible to mechanical stress and lead to a premature degradation of red cells. We now define a deficiency in Cdk6 kinase activity as the cause of a novel form of anemia characterized by enhanced mechanical instability of erythrocytes.

Mice lacking *Cdk6* or its kinase activity (*Cdk6*$^{K43M/K43M}$) suffer from a mild form of anemia with enhanced numbers of Ter119^{+} cells in the bone marrow and increased numbers of reticulocytes in the peripheral blood. This observation is in line with enhanced erythropoiesis, which may be viewed as the organism's attempt to compensate for the enhanced loss of erythrocytes. We did not detect any reduction of erythroblast proliferation or differentiation in *in vitro* systems that mimic stress erythropoiesis or when mice were treated with phenylhydrazine.

Cdk6 does not have a critical role in cell proliferation, since its function can be performed in its absence by Cdk4. Differentiation along the erythroid lineage is largely unaltered by the elimination of Cdk6. This finding is in contrast to a report that Cdk6 is involved in the differentiation of the murine erythroleukemia cell line, MEL. The apparent discrepancy probably relates to the different experimental systems used. Transformed cell lines harbor multi-

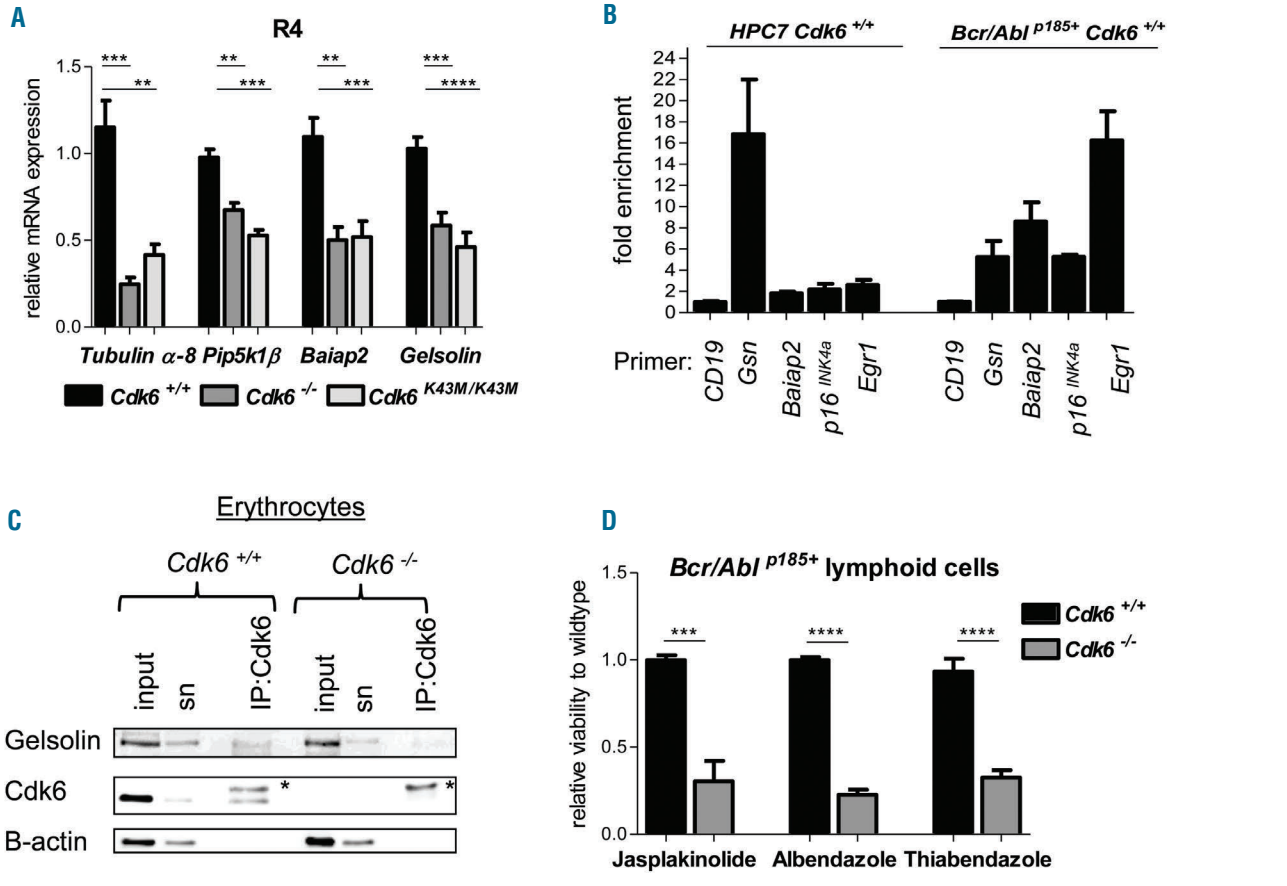


Figure 6. ***Cdk6* regulates transcription of genes involved in cytoskeleton organization in a kinase-dependent manner.** (A) Gene expression was analyzed by quantitative reverse transcriptase polymerase chain reaction in region R4 (orthochromatophilic erythroblasts) of indicated bone marrow. Relative expression levels were normalized to *Rplp0* mRNA. Statistical analysis was carried out using a two-tailed unpaired Student *t* test. Error bars indicate ± SEM (n≥6 per group; ***P*<0.01; ****P*<0.001; *****P*<0.0001). (B) Chromatin immunoprecipitation experiments were performed in the mouse HPC7 *Cdk6*$^{+/+}$ progenitor cell line and *Bcr/Abl*$^{p185+}$ *Cdk6*$^{+/+}$ lymphoid cells. Protein-DNA complexes were immunoprecipitated using home-made sera against Cdk6 and analyzed by quantitative polymerase chain reaction for the presence on the indicated promoter regions. *p16*^{INK4a} and *Egr1* promoter regions served as positive controls. Bar graphs depict fold enrichment over a negative region downstream of *CD19* as described in the Methods section. (C) Lysates from mature erythrocytes were subjected to Cdk6 immunoprecipitation (IP) and blotted with an anti-gelsolin antibody. The asterisk indicates an unspecific cross-reacting band. Beta-actin served as loading control. sn: supernatant. (D) Viability measurements upon treatment with an actin-specific agent Jasplakinolide (4 nM) and microtubule depolymerizing agents albendazole (1 μM) and thiabendazole (10 μM) for 72 h were conducted using the CellTiterGlo Viability Assay. The analysis was carried out in triplicate. A two-tailed unpaired Student *t* test was used for the statistical comparison. Error bars indicate ± SEM (****P*<0.001; *****P*<0.0001). (E) The absorbance of hemoglobin supernatant from indicated mature erythrocytes at 540 nm after a constant shear stress is depicted. Statistical analysis was carried out using a two-tailed unpaired Student *t* test. Error bars indicate ± SEM (n=12 per group; *****P*<0.0001; n.s.: not significant).

ple mutations that may interfere with normal or regular differentiation and so may not mimic the situation in untransformed cells. For example, loss of CDK6 in MLL-transformed myeloid acute myelogenous leukemia cells induces differentiation[35] while the myeloid compartment in *Cdk6*-deficient mice is normal and contains cells at all stages of maturation.[4] Signaling and transcriptional control are rewired in transformed cells so that molecules that are not required in non-transformed cells may become important for differentiation or proliferation.

Erythroblast protein expression changes during differentiation. Certain proteins are degraded or removed with the nucleus at enucleation or by vesiculation during reticulocyte maturation.[36–38] Most proteins are degraded during development. Levels of Cdk4 decrease dramatically and the protein is not detectable in mature erythrocytes, although Cdk6 is still present. Together with the observation that *Cdk6*- but not *Cdk4*-deficient mice[5,6] suffer from anemia, this provides strong evidence that Cdk6 has a function in murine erythrocytes that cannot be performed by Cdk4. Interestingly, in the absence of Cdk6, mature erythrocytes do contain detectable levels of Cdk4. This paradoxical finding can be explained by postulating that in the absence of Cdk6, Cdk4 can interact with cytoskeletal structures usually occupied by Cdk6, thereby being at least partially protected from degradation, although the interaction may not have any functional consequence.

Previous studies indicated that expression of cell-cycle components correlate with variations in size and cell divisions during erythropoiesis.[39] A direct role has been assigned to cyclin D3: its levels orchestrate the number of cell divisions during terminal erythropoiesis, thereby controlling the number and size of erythrocyte progeny.[40] In contrast, proliferation and differentiation of erythrocytes are not impaired in mice lacking *Cdk6* and mature erythrocytes contain Cdk6. We thus speculated that the anemic phenotype might be linked to alterations in the cytoskeleton. Cdk6 has been reported to associate with the cytoskeleton in astrocytes, where forced overexpression of Cdk6 induces changes to the cytoskeletal organization.[10,17] Staining of erythroid cells for F-actin revealed a dependence on the presence of Cdk6, with *Cdk6*-deficiency associated with markedly lower levels of F-actin. A number of clinical conditions have been linked to impaired F-actin formation[18,41,42] and these may be associated with a shortened half-life of erythrocytes in the peripheral blood. We found an altered F:G-actin composition in transformed lymphoid cells, indicating that the role of Cdk6 in actin remodeling is not restricted to erythroid cells. A possible mechanism is suggested by the observation that Cdk6 interacts in mature erythrocytes with gelsolin, a known regulator of actin remodeling.[19]

The cytoskeleton of erythrocytes faces particular challenges. Erythrocytes must pass through narrow capillaries, placing special requirements on the stability and flexibility of the cytoskeleton.[18] Our results point to a further unique feature of the erythrocyte cytoskeleton, namely its dependence on Cdk6 for structural integrity and flexibility. Cdk6 is tethered to the erythrocyte cytoskeleton and this finding is consistent with the localization of the protein when it is overexpressed in astrocytes.[17]

We and others have shown that Cdk6 directly regulates transcription in both kinase-dependent and kinase-independent manners, interacting with a variety of transcription factors including Stat and AP-1 as well as with nuclear factor-κB.[14–16,43] We now report that Cdk6 promotes the transcription of genes involved in cytoskeletal organization. The function depends on Cdk6 kinase activity: upon loss of Cdk6 kinase activity in erythroid progenitors (R4) there is a significant decrease in mRNA levels of *Tubulin alpha-8* implicated in microtubule assembly and *Baiap2*, *Gelsolin* and *Pip5k1b* involved in actin dynamics. BAIAP2 participates in F-actin rearrangements when activated by small GTPases.[21] Loss of gelsolin in mice has been shown to change the balance between polymerized and depolymerized actin in red blood cells.[19] PIP5K1B has a function in the dynamics of the actin cytoskeleton[44] and PIP5 kinases synthesize phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$], which binds to gelsolin and thereby promote actin polymerization.[45–47] Loss of Cdk6 kinase activity thus causes impaired actin remodeling, which is likely to result in increased fragility of mature erythrocytes.[48] Interestingly, mass spectrometry analysis of both erythroid and lymphoid cells confirmed that Cdk6 interacts with a number of proteins involved in cytoskeletal organization, suggesting a global role of Cdk6 in cytoskeletal integrity.

Two sets of experiments show that our results have functional significance. First, loss of *Cdk6* causes mechanical instability of red blood cells in the shear test, which mimics the entry of erythrocytes into the narrow capillaries of the body. Secondly, *Cdk6*-deficient cells are more sensitive to actin and microtubule inhibitors.

Our work defines Cdk6 as a unique member of the Cdk family with a potential dual function for the cytoskeleton. In erythroid cells, Cdk6 affects cytoskeletal stability by transcriptionally regulating a set of genes that control cytoskeletal organization. This function depends on Cdk6 kinase activity. Furthermore, Cdk6 has a direct structural role in erythrocytes. It is tethered to the cytoskeleton, where it may phosphorylate unknown proteins, be a stabilizing anchoring factor or simply be protected from degradation. Further research is needed to unravel the mechanisms behind this function and its consequences.

The phenotype of $Cdk6^{-/-}$ mice is recapitulated in $Cdk6^{K43M/K43M}$ animals. This finding may have consequences for therapies that target CDK6 kinase activity over a longer time. Inhibitors of the CDK4/6 kinases are being tested for use in the treatment of many forms of cancer.[49] Our findings provide a possible explanation for the observation that patients receiving inhibitors of CDK4/6 kinases are prone to reduced erythroid stability which contributes to anemia.[50]

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