Replication stress caused by low MCM expression limits fetal erythropoiesis and hematopoietic stem cell functionality

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Replicative stress during embryonic development influences ageing and predisposition to disease in adults. A protective mechanism against replicative stress is provided by the licensing of thousands of origins in G1 that are not necessarily activated in the subsequent S-phase. These ‘dormant’ origins provide a backup in the presence of stalled forks and may confer flexibility to the replication program in specific cell types during differentiation, a role that has remained unexplored. Here we show, using a mouse strain with hypomorphic expression of the origin licensing factor mini-chromosome maintenance (MCM)3 that limiting origin licensing in vivo affects the functionality of hematopoietic stem cells and the differentiation of rapidly-dividing erythrocyte precursors. Mcm3-deficient erythroblasts display aberrant DNA replication patterns and fail to complete maturation, causing lethal anemia. Our results indicate that hematopoietic progenitors are particularly sensitive to replication stress, and full origin licensing ensures their correct differentiation and functionality.

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The process of genomic duplication starts at replication origins, which are licensed in the G1 phase of the cell division cycle, several hours before their activation in S phase. The licensing process is led by the origin recognition complex (ORC), cell division cycle 6 (CDC6) and Cdc10-dependent transcript 1 (CDT1) proteins, which cooperate to engage the mini-chromosome maintenance (MCM) complex with the DNA. MCM, composed by essential subunits MCM2-7, displays DNA helicase activity and becomes part of the replisome machinery (reviewed in references 12–14). Defective control of DNA replication causes 'replicative stress' (RS), which is the underlying cause of several developmental diseases. Mutations in ORC, CDC6 and CDT1 genes are related to Meier-Gorlin syndrome, a type of dwarfism 3–5, and mutations in MCM4 are linked to growth retardation, adrenal insufficiency and natural killer cell deficiency 6. Impaired MCM function also increases cancer susceptibility 7–13 (reviewed in references 14,15).

MCM complexes are normally loaded onto DNA in excess relative to the number of origins that fire during the S phase (reviewed in reference 16). One function of the surplus of MCM is to license dormant origins that may be activated in response to stalled or collapsed forks, providing a rescue mechanism under RS 17–19. Another possible function for the high number of licensed origins, which remains largely underexplored, is to provide flexibility to the replication process during early embryonic development 20,21 or in cell differentiation contexts that require the activation or shut-off of specific origins 22–24 (reviewed in references 25,26).

To investigate the protective effects of MCM against RS in vivo, a new Mcm3 conditional knockout (KO) mouse model was designed. The targeted allele, Mcm3-Lox, was hypomorphically expressed and caused embryonic lethality in homozygosity, which could be partially alleviated in a genetic background with enhanced resistance to RS. Our results show how a full complement of MCM proteins is specifically required to preserve the functionality of hematopoietic stem cells (HSCs) and the proper differentiation and maturation of erythrocytes in the developing embryo.

**Results**

A hypomorphic Mcm3 allele causes embryonic lethality. A modified mouse Mcm3 allele was designed with loxP sites flanking exons 14–17 and a luciferase reporter inserted at the 3′ UTR under the control of an IRES element. The resultant allele (Mcm3-Lox) was intended as a conditional KO, as Mcm3 expression could be ablated with Cre recombinase (Fig. 1a and Supplementary Fig. 1A,B). In addition, expression of Mcm3-Lox could be monitored by the bioluminescence activity associated to luciferase expression. Mcm3+/Lox mice were born at Mendelian rates, and Mcm3-Lox expression in the skin and several internal organs was confirmed by luciferase activity after the administration of luciferin (Fig. 1b and Supplementary Fig. 1C). Unexpectedly, virtually no Mcm3Lox/Lox individuals were born after extensive breeding of Mcm3+/Lox mice (1 in 314; Fig. 1c). Mcm3Lox/Lox embryos survived until E16.5–E18.5 but were noticeably smaller than wild-type (wt) or Mcm3+/+ littermates and their pale appearance suggested an anemic phenotype (Fig. 1d). Mcm3+/Lox and Mcm3Lox/Lox embryonic fibroblasts (MEFs) could be obtained and maintained in culture.

To determine the impact of the Mcm3-Lox modification on gene function, mRNA levels were reduced to approximately 70% in Mcm3+/Lox and 30% in Mcm3Lox/Lox MEFs, relative to the levels in Mcm3+/+ MEFs (Fig. 1e), and consistent reductions in MCM3 protein were observed in whole cell extracts (Fig. 1f; compare lanes 3–5–7). These results indicate that the Mcm3-Lox allele is hypomorphically expressed, presumably due to the modifications introduced in the 3′ UTR. This level of reduction in MCM3 did not significantly affect the cellular concentration of other MCM subunits (Fig. 1f).

At least in primary embryonic fibroblasts, downregulation of Mcm3 expression was compatible with DNA replication and cell proliferation (Supplementary Fig. 3A,B). Analyses of inter-origin distances (IOD) using stretched fibers revealed a similar frequency of origin activity between Mcm3+/+ and Mcm3Lox/Lox MEFs in normal growth conditions (median IOD values of 118 and 116 Kb, respectively; Supplementary Fig. 3C). Upon challenging fork progression with aphidicolin, the IOD was shortened by 37% in Mcm3+/+ MEFs, as a consequence of the activation of dormant origins (median IOD 73.8 Kb; Supplementary Fig. 3C). In Mcm3Lox/Lox MEFs, the reduction in IOD was limited to 24% (median IOD 87.5 Kb), reflecting a partial loss of back-up origins but also underscoring the large excess of origins licensed in G1, as even after the marked reduction in MCM3 protein concentration, a reservoir of additional origins were activated when needed. After several days in culture, Mcm3Lox/Lox MEFs displayed γH2AX foci, a common marker of DNA damage associated to RS (Supplementary Fig. 3D).

Mcm3-deficient mice are prone to haematological neoplasia. In contrast to Mcm3Lox/Lox heterozygous Mcm3+/Lox mice were viable and could be crossed with a CMV-Cre strain expressing Cre recombinase to generate viable Mcm3+/− mice. As expected, breeding between Mcm3+/− mice did not produce any Mcm3−/− offspring (Fig. 1g). All previous attempts at making MCM-null models displayed pre-implantation lethality 8–10. When cohorts of Mcm3+/− and Mcm3−/Lox mice were established for longevity studies, both groups presented a slight but significant reduction in lifespan (Fig. 1h) and an increased incidence of tumors, mainly lymphomas originated in the mesenteric lymph nodes that in multiple cases infiltrated to other organs. These tumors are not uncommon in aged mice but normally have a later onset and lower invasiveness (Supplementary Tables 1–3).

DNA damage and incomplete erythropoiesis in the fetal liver. We next focused on the tissues that might be responsible for the lethality observed in Mcm3Lox/Lox embryos, which by mid-late gestation (E14.5–18.5) were consistently smaller than their Mcm3+/+ or Mcm3+/Lox counterparts. In control E16.5 embryos, MCM3 protein is readily detected by immunohistochemistry (IHC) in most tissues, particularly liver, lung, thymus, brown adipose tissue and the subventricular zone of the brain. In Mcm3Lox/Lox embryos, the strong MCM3 staining in the liver was markedly reduced while γH2AX staining became apparent (Fig. 2). Mcm3 mRNA and protein levels confirmed the downregulation of Mcm3 expression in fetal liver extracts (Supplementary Fig. 4A,B). Global levels of cell proliferation were not affected, as monitored by Ki67 IHC staining (Supplementary Fig. 4C).

The liver is the major site of fetal hematopoiesis from E11 until shortly before birth, when this process becomes established in the bone marrow. To evaluate the impact of Mcm3 downregulation in hematopoiesis, specific blood lineages were detected by IHC in mid-late gestation embryos. This approach did not reveal major differences in the abundance of mature B-lymphocytes (stained with Pax-5) and megakaryocytes (stained with FITC) in the fetal
liver, nor T-lymphocytes (stained with CD3) in the fetal thymus of Mcm3lox/Lox embryos compared to their Mcm3+/+ or Mcm3+/Lox counterparts (Supplementary Fig. 5). In contrast, the staining with erythocyte marker Ter119 was much reduced in the liver of Mcm3lox/Lox embryos (Fig. 3a). Peripheral blood from Mcm3-deficient E14.5 embryos displayed a marked decrease in red blood cells (RBCs), haemoglobin concentration and hematocrit (Fig. 3b), all consistent with a phenotype of fetal anemia. Giemsa staining of peripheral blood cells revealed a large accumulation of immature erythroblasts (EBs) with uncompacted nuclei, as well as the presence of micronuclei that are indicative of genomic instability (Fig. 3c,d).

Erythrocyte differentiation is coupled to 3–5 rounds of DNA replication and cell division. Based on the expression of surface markers CD71 and Ter119, EBs can be separated by flow cytometry in consecutive differentiation stages, pro-EBs (located in 'region' R1); early basophilic EBs (baso-EBs; R2); early and late baso-EBs (R3); chromatophilic/orthochromatophilic EBs (chr-EBs/orth-EBs; R4); and late orth-EBs/reticulocytes (R5). In agreement with the analyses of embryo peripheral blood, the fetal liver of Mcm3lox/Lox embryos displayed an accumulation of pro-EBs and early baso-EBs, and a concomitant reduction in chr-EBs, orth-EBs and mature reticulocytes (Fig. 3e). Activated caspase 3 was detected in a
fraction of R3 Mcm3-deficient EBs, indicating apoptotic events (Supplementary Fig. 6).

**Impaired erythrocyte maturation upon transplantation.** The fetal liver contains HSCs capable of reconstituting the entire hematopoietic system. In order to compare the maturation ability of wild-type and Mcm3-deficient cells towards the erythroid lineage, fetal liver cells (E14.5) derived from wild-type or Mcm3-deficient embryos were transplanted into lethally-irradiated recipient mice, in competition (1:1 ratio) with bone marrow cells that constitutively express a Tomato (Tom) fluorescent marker. Two months after transplantation, the contribution of control Mcm3+/+ cells (Tom-negative) towards mature RBCs in peripheral blood was approximately 50% in the chimera (Fig. 4b). In contrast, the contribution of Mcm3-deficient cells (Tom-negative) towards RBCs was much reduced, with Mcm3-proficient cells (Tom-positive) taking over most of the RBC population (Fig. 4b). These results confirm that EBs with reduced expression of Mcm3 do not mature properly under steady-state conditions.

Chimaeric Mcm3+/+ (Tom+/+) / Mcm3Lox/Lox (Tom−) mice were also used to monitor the acute response to erythropoietic stress induced by phlebotomy (Fig. 4c). In this setting, cells in the spleen with Ter119 high content can be analyzed using the forward scatter parameter (FSC) and CD71 to resolve three EB subpopulations previously labelled as EryA, EryB and EryC. EryA (Ter119 high CD71 high FSC high) correspond to baso-EBs; EryB (Ter119 high CD71 high FSC low) correspond to late baso-EBs and polychromatic-EBs; EryC (Ter119 high CD71 low FSC low) are ortho-EBs and reticulocytes. In response to stress, the EryA and EryB subpopulations are rapidly increased. Both Mcm3-competent and Mcm3-deficient EBs reacted to stress, as indicated by the accumulation of splenic EryA and EryB progenitors (Fig. 4d,e). However, the accumulation of EryA in Mcm3-deficient cells was almost 3-fold higher than in Mcm3+/+ cells, suggesting that the rapid RBC maturation required under stress conditions is also delayed or impaired (Fig. 4e, right).

### Aberrant DNA replication during erythrocyte maturation.

To monitor whether inefficient erythrocyte maturation was related to DNA replication and cell cycle progression, fetal liver cells obtained from Mcm3+/+, Mcm3+/Lox and Mcm3Lox/Lox embryos were sorted at the R1-R4 stages (R5 was not included as most cells have started the enucleation process). Interestingly, Mcm3Lox/Lox baso-EBs (R3) and chr-EBs (R4) displayed a prominent accumulation in S phase, indicative of problems to complete DNA replication (Fig. 5a). High levels of γH2AX and a higher frequency of fork asymmetry (indicative of stalled forks) were detected in Mcm3Lox/Lox early EBs, consistent with RS interfering with RBC maturation (Fig. 5b,c).

Next, we analyzed the patterns of origin activity in EB populations sorted from embryonic livers using stretched DNA fibers labelled with thymidine analogs chloro-deoxyuridine (CldU) and iodo-deoxyuridine (IdU). These analyses revealed a dynamic pattern in which more origins are activated as EBs progress in the differentiation pathway. The median IOD was progressively reduced from approximately 100 Kb in pro-EB (R1) to 74 Kb in early baso-EBs (R2), 69 Kb in late baso-EBs (R3) and 56 Kb in ortho-EBs (R4; Fig. 5d, left). The length of DNA fibers was similar in all populations, ruling out indirect effects on IOD measurements (Supplementary Fig. 7). Furthermore, the tendency towards more origin activity was confirmed by measuring origin density (Fig. 5d, right and Table 1). Interestingly, the patterns of origin activity were altered in Mcm3-deficient EBs, which displayed lower IOD values and slightly higher origin density (Fig. 5d and Table 1). As discussed below, this behaviour likely reflects the increased pressure on early EBs to replicate as a compensatory mechanism for the lack of mature RBCs.

### Mcm3Lox/Lox lethality can be rescued by CHK1 overexpression.

Because our results strongly point to RS as the underlying cause for incomplete RBC maturation, we considered the possibility that the embryonic lethality of the Mcm3Lox/Lox genotype could be alleviated in a background with higher tolerance to RS, such as the recently described strain carrying an extra copy of checkpoint kinase CHK1 (ref. 31). Mcm3+/Lox, Chk1+/+ mice...
were generated by direct intercrossing between both strains, and further breeding of Mcm3\(^+/\)Lox, Chk1\(^{+/+}\)tg mice resulted in Mcm3\(^{Lox/Lox}\) individuals that successfully completed gestation, despite having low expression levels of Mcm3 (Supplementary Fig. 8). A few Mcm3\(^{Lox/Lox}\) individuals were born without the extra Chk1 allele, indicating that the change in genetic background (now mixed C57BL/6-CD1) also contributed to survival of the Mcm3\(^{Lox/Lox}\) embryos. Independently of the effect of the mixed background, the birth rate was biased towards Mcm3\(^{Lox/Lox}\), Chk1\(^{+/+}\)tg progeny. This partial rescue of embryonic lethality provides further genetic evidence that RS caused by Mcm3 downregulation is the reason for the phenotypes observed in the Mcm3\(^{Lox/Lox}\) strain.

**Impaired fitness of Mcm3\(^{Lox/Lox}\) hematopoietic stem cells.** A recent report has linked RS to the limited functionality observed in aged HSCs\(^3^2\). Our Mcm3-deficient model provided a proper genetic tool to validate this concept, as Mcm3-deficient fetal HSCs should also be affected. Fetal Mcm3\(^{Lox/Lox}\) LSK cells (Lin\(^{-}\)/cKit\(^{+}\)/Sca1\(^{+}\)/Flk2\(^{-}\)/CD48\(^{-}\)/CD150\(^{+}\)) and multipotent progenitor cells (MPPs: Lin\(^{-}\)/cKit\(^{+}\)/Sca1\(^{+}\)/Flk2\(^{+}\)), had lower amounts of Mcm3 protein and higher levels of RS marker γH2AX (Fig. 6a). Intriguingly, single-cell tracking of cell division revealed slightly faster proliferation kinetics of Mcm3\(^{Lox/Lox}\) than Mcm3\(^{+/+}\) fetal HSCs (Fig. 6b). This could reflect a pressure to proliferate and differentiate into MPPs, as it has been described for adult HSCs in the presence of DNA damage\(^3^3\). Consisting with this notion, immunophenotyping analyses revealed a two-fold increase in the concentration of LSK cells in Mcm3\(^{Lox/Lox}\) embryos, caused by the accumulation of MPPs (Fig. 6c).

To directly test their functionality, fetal HSCs were isolated from E14.5-E16.5 CD45.2 C57BL/6 Mcm3\(^{+/+}\), Mcm3\(^{+/Lox}\) and Mcm3\(^{Lox/Lox}\) embryos and transplanted into lethally irradiated congenic CD45.1 C57BL/6 recipient mice. Four months after transplantation, recipient mice displayed decreased Mcm3\(^{Lox/Lox}\) chimerism in all hematopoietic organs (Fig. 6d). Because formation of an adult HSC compartment was observed (Fig. 6d, right), donor-derived HSCs were re-isolated from the bone marrow of recipient mice to evaluate their proliferation potential in vitro and in vivo. Mcm3-deficient adult HSCs displayed RS markers γH2AX and RPA (Fig. 6e) and inefficient colony formation in methylcellulose in the presence of aphidicolin.
Figure 4 | Impaired RBC maturation during steady-state or stress erythropoiesis. (a) Experimental design of a competitive transplantation assay between Mcm3+/+ and Mcm3Lox/Lox fetal liver (FL; E14.5) cells and bone marrow (BM) cells of a tool strain constitutively expressing red fluorescent Tomato (Tom) protein, with wild-type Mcm3 levels. In each case, cells were transplanted at 1:1 ratio. (b) Quantification of the RBC (Ter119-positive) chimerism in peripheral blood of recipient mice, 2 months post-transplantation. Histograms show average ± s.d.; n = 4 mice transplanted with Mcm3+/+ cells and n = 4 mice transplanted with Mcm3Lox/Lox cells. P-values were calculated by Fisher’s test (**P < 0.01, NS, not significant). (c) Schematic of a phlebotomy assay in chimaeric mice to monitor the erythropoietic stress response. (d) Examples of flow cytometry analyses of splenic EryA, EryB and EryC precursors (abbreviated A, B, C), in control mice (CTR) or mice subjected to phlebotomy (PHL). Analyses of EryA, EryB and EryC was carried out four days after the bleeding, differentiating between Tom+ and Tom− cells. (e) Left, histograms showing the percentage of EryA, EryB and EryC populations relative to control mice (n = 2 CTR, n = 4 PHL mice). P-values were calculated by Fisher’s test (**P < 0.001, **P < 0.01). Right, histogram showing the fold-change variation in Tom+ (red) or Tom− (white) EryA, EryB and EryC populations induced by phlebotomy.

Discussion

Mcm2-7 are essential genes that encode key components of the main DNA helicase involved in genome replication. Mcm2-7 are highly expressed in proliferating cells, and DNA replication can still occur in cell lines after a significant reduction (>90% in some cases) in the cellular concentration of MCM protein complexes17-19. This tolerance to MCM downregulation is related to the fact that MCM complexes are engaged with DNA in large excess relative to the number of replication forks normally established during S phase. The surplus of MCM complexes license dormant origins that are activated only as a rescue mechanism when DNA replication is disrupted (reviewed in reference34). The use of dormant origins in vivo is well documented in the mouse, and MCM downregulation beyond ~2/3 of its physiological levels causes embryonic lethality or promotes tumorigenesis in adults2-13. Therefore, it is likely that certain cell types in the developing embryo, such as stem and progenitor cells, are particularly sensitive to RS induced by low MCM levels.

We have tested the hypothesis that different cell types may have different requirements for MCM concentration using a novel strain with hypomorphic Mcm3 expression. While Mcm3Lox/Lox MEFs proliferated and replicated DNA with approximately 1/3 of the normal concentration of MCM3 protein, a similar reduction severely impaired hematopoietic progenitors, indicating a stricter requirement for origin licensing in the latter. In mid-gestation, hematopoiesis in the fetal liver is largely geared towards the production of RBCs to guarantee oxygen delivery to the rapidly growing embryo. Interestingly, erythroid precursors undergo several rounds of DNA replication and cell division during terminal differentiation, and genetic models that ablate cell cycle regulators such as Rb, E2F4, E2F8 or D-cyclins frequently result in embryonic anemia35-38. While D-Cyclin/CDK and Rb/E2F constitute the axis required for erythrocyte maturation in steady-state conditions and in response to erythropoietic stress. If MCM levels drop to

(Fig. 6f). Furthermore, when subjected to a major replication challenge such as a secondary transplantation, Mcm3Lox/Lox adult HSCs were drastically affected in their ability to self-renew and regenerate the blood system (Fig. 6g).

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approximately 1/3 of its normal concentration, the severity of the anemia causes embryonic lethality in the C57BL/6 genetic background. As reported in other mouse models such as the Mcm4-chaos mutant8,11,12 or the Rif1 KO mouse39, Mcm3Lox/Lox embryonic lethality was partially alleviated in a mixed C57BL/6-CD1 background and it was further rescued by overexpression of CHK1 kinase, reinforcing the connection between RS and the phenotypes observed.

To our knowledge, the single-molecule analyses of DNA replication in EB precursors isolated from the fetal liver provide the first evidence that the program of DNA replication undergoes active changes during the physiological maturation of mammalian erythrocytes. As pro-EBs proliferate and differentiate into mature reticulocytes, their replication program requires the progressive activation of more origins. This observation has interesting antecedents: chicken erythrocytic progenitors forced

Figure 5 | Aberrant DNA replication patterns in differentiating EBs. (a) DNA content of EBs in R1-R4 differentiation stages. Three examples of each genotype are shown. (b) γH2AX intensity in Mcm3+/+, Mcm3+/+Lox and Mcm3Lox/Lox R3 EBs (> 300 nuclei scored per condition). Data are representative of 3 independent experiments. P-values were calculated by Mann-Whitney test (**P<0.001). (c) Box plots showing the values of fork asymmetry in Mcm3+/+ and Mcm3Lox/Lox EBs sorted in R1 and R3 stages. >180 bidirectional forks were counted per condition. Statistical analysis was done with Mann-Whitney rank sum test (**P<0.001). (d) Left, IOD values (data distribution and median value) of Mcm3+/+, Mcm3+/+Lox and Mcm3Lox/Lox EBs at the indicated (R1-R4) maturation stage. >100 IOD values were scored for each condition. P-values were calculated using Mann-Whitney test (**P<0.001; NS, not significant). Right, origin density expressed by number of origins/Mb of DNA was estimated as indicated in Materials and Methods. See also Table 1.

Table 1 | Origin density in Mcm3+/+ and Mcm3-deficient EBs.

|       | R1  | R2  | R3  | R4  |
|-------|-----|-----|-----|-----|
|       | +/+ | L/L | +/+ | L/L | +/+ | L/L | +/+ | L/L |
| n. oris | 327 | 382 | 325 | 344 | 285 | 292 | 285 | 250 |
| DNA, Mb | 141.3 | 118.4 | 110.9 | 108.7 | 89.91 | 84.24 | 85.29 | 69.10 |
| ori, Mb | 2.32 | 3.23 | 2.93 | 3.16 | 3.17 | 3.47 | 3.34 | 3.62 |

Proliferating EBs derived from the fetal liver of Mcm3+/+ or Mcm3Lox/Lox (L/L) embryos were labeled with CldU and IdU and isolated at different maturation stages (R1-R4) by flow cytometry after CD71/Ter119 staining. Origin density was calculated by measuring the number of origin structures in multiple microscopy fields, until a minimum of 250 origins were scored per condition, and the total length of DNA in every fiber containing at least one replication structure.
Fisher's test (***P < 0.001) of treated cells (DMSO) and expressed as fold change compared to Mcm3+Mcm3Lox/Lox HSCs isolated from the BM. Scale bar, 10 μm. Right panels, quantification of McM3 or γH2AX staining intensity, using Definiens software on confocal microscopy images. Red lines indicate the median nuclear intensity (> 800 nuclei scored per condition). P-values were calculated by Mann-Whitney test (** * P < 0.001). (b) Single-cell tracking of the first cell division of cultured fetal Mcm3+/+ or Mcm3Lox/Lox HSCs. Representation shows the average ± s.d. of 4 independent experiments. (c) Histograms showing the percentage of LSK, MPP and HSCs in Mcm3+/+, Mcm3+/Lox and Mcm3Lox/Lox embryonic liver tissue. Median values ± s.d. are shown (n = 7 embryos for each genotype). P-values were calculated by Fisher's test (** * P < 0.05; NS, not significant). (d) HSC engraftment results at 4 months post-transplantation. Middle histograms show the percentage of donor-derived chimerism in the bone marrow (BM), spleen and peripheral blood. Right histograms show the percentage of chimerism in the adult HSC compartment. Quantification shows average ± s.d. of three independent experiments. P-values were calculated by Fisher's test (** * P < 0.001; * P < 0.05; NS, not significant). (e) Confocal microscopy images of γH2AX and RPA staining in donor-derived, cycling adult Mcm3+/+ or Mcm3Lox/Lox HSCs isolated from the BM. Scale bar, 10 μm. (f) Histogram showing the average number of colonies (± s.d.) formed 7 days after plating Mcm3+/+ or Mcm3Lox/Lox HSCs in methylcellulose, in the absence or presence of low-dose aphidicolin (50 ng ml⁻¹). Results are normalized for vehicle-treated cells (DMSO) and expressed as fold change compared to Mcm3+/+ HSCs. 3 independent experiments were conducted and included in the quantification. P-values were calculated using Fisher’s test (** P < 0.05; NS, not significant). (g) Donor-derived adult Mcm3+/+ and Mcm3Lox/Lox HSCs were re-isolated and transplanted into lethally irradiated secondary recipients (500 HSCs per mouse; 5 mice per group). Histogram shows the percentage of donor-derived chimerism in peripheral blood, assessed 1–4 months post-transplantation. Data are mean values ± s.d. P-values were calculated using Fisher’s test (** * P < 0.001).

to differentiate ex vivo display a broadening in origin usage in the β-globin locus. Also, origin firing is enhanced in murine erythroleukemia cells derived from transformed pro-EBs and forced to differentiate in culture. It is conceivable that rapid DNA replication, driven from an increasingly high number of origins, is related to the rapid loss of DNA methylation marks observed during mouse erythropoiesis. These patterns of DNA replication were altered in Mcm3-deficient pro-EBs, which displayed a higher frequency of origin activation and asymmetric forks from the start of their differentiation program. Later in differentiation, baso-EBs and chr-EBs were not capable of completing DNA replication, accumulated in S phase and activated the apoptotic program, thus preventing erythrocyte maturation. This likely triggers a compensation mechanism that pressures pro-EBs to replicate and proliferate, contributing to the aberrant replication patterns. Fork asymmetry
reflects improper coordination between fork pairs and may also trigger the activation of compensatory origins. The fact that proEBs were capable of activating extra origins after Mcm3 downregulation underscores the fact that a very large excess of MCM proteins associate with DNA during G1.

Mid gestation is also the time of HSCs expansion in the fetal liver. At this stage, HSCs divide very rapidly (reviewed in reference 43), which could also make them vulnerable to RS. The liver. At this stage, HSCs divide very rapidly (reviewed in references 49, 50). Interestingly, a comparison of gene expression profiles of young versus old adult HSCs revealed that Mcm3-Lox/Lox HSCs were re-isolated from the bone marrow of recipient mice and tested in secondary transplantations. These EBs were capable of activating extra origins after Mcm3 downregulation underscores the fact that a very large excess of MCM proteins associate with DNA during G1.

In summary, Mcm3 downregulation during embryonic development caused RS in hematopoietic progenitors leading to fetal anemia; in adults, it reduced life expectancy and promoted lymphomagenesis. Thus, future therapies designed to modulate RS are a promising way to fight ageing and hematopoietic malignancies.

Methods

Generation of Mcm3+/Lox mouse strain. A targeting vector was designed that included an IRES-EGFP-luciferase reporter cassette after the Mcm3 stop codon located at exon 17, two loxP sites flanking Mcm3 exons 14 to 17, and a FRT-flanked neomycin-resistance cassette for selection. The linearized vector was electroporated into 129Sv/Pas ES cells, and its genomic integration by recombination was screened by PCR and Southern blotting. Verified transgenic ES clones (Mcm3-Lox-Neo) were microinjected into C57BL/6J blastocysts and germline transmission was screened by PCR and Southern blotting. Verified transgenic ES clones were microinjected into C57BL/6J blastocysts and germline transmission was confirmed. Cell nuclei were stained with DAPI (Sigma). Images were acquired either in a Leica-TCS SP5X confocal microscope, with a HCX PL APO 20x objective using LAS AF software or in an Opera High-Content Screening System (PerkinElmer) with an APO 20 × 0.7 NA water-immersion objective using Acapella software (PerkinElmer). To estimate the number of RPA foci-positive cells or γH2AX foci-positive cells, 100–200 or 250–500 cells were scored respectively in each condition. To measure intensity of MCM3 or γH2AX in erythroid populations, images were acquired from each cell, nuclei were marked by DAPI staining and protein immunostaining intensity was measured within the nuclei.

Single-molecule analysis of DNA replication. Exponentially growing MEFs or single-cell suspensions of fetal liver were pulse-labeled with 50 μM EdU (20 min) followed by 250 μM IdU (20 min). Labeled cells were harvested and resuspended in 0.2 M Tris pH 7.4, 50 μM EDTA and 0.5% SDS. Stretched DNA fibers were prepared as described26. For immunodetection of labeled tracks, fibers were incubated with primary antibodies for 1 h at RT and the corresponding secondary antibodies for 1 h at RT, in a humidity chamber. Fiber was stained with anti-ssDNA to assess fiber integrity. Fiber images were obtained in a DM6000 B Leica microscope with an HCX PL APO 40 × 0.75 NA objective. The conversion factor used was 1 μm = 2.59 kbp. In each assay, >300 individual tracks were measured for FR estimation, >100 fibers containing two or more origins were analyzed for IOD estimation, >200 labeled fibers were counted for fiber length and origin density, and >180 bidirectional forks were counted for fork symmetry.

Histochemistry and immunohistochemistry (IHC). Embryos, normal tissues and tumour samples were fixed in 10% buffered formalin (Sigma) and embedded in paraffin using standard procedures. For histopathological studies, 3 μm sections were stained with haematoxylin and eosin (H&E). The following antibodies were used for IHC analysis: Mcm3 (generated in rabbits immunized with synthetic polyclonal antigen conjugated to KLH; KPL; rabbit serum was used at 1:500 dilution), K67 (Dako, 1:50), γH2AX (Millipore, 05-636: 1:100), pS15-p53 (Cell Signaling, 8298; 1:200), Ter119 (BD, 550565: 1:50), Pax5 (Santa Cruz Biotechnology, sc-1974: 1:50), CD3 (Santa Cruz Biotechnology, sc-1127: 1:50) and FITC (DAKO, A0082: 1:200). Tissue slides were imaged for analysis using a Mirax scanner (Carl Zeiss) and equivalent areas per tissue and group were analyzed using AxioVision digital image processing software (Carl Zeiss). Areas of positive staining were normalized to the total analyzed area.

Fluorescence activated cell sorting of hematopoietic cells. To prepare fetal liver cell suspensions, E14.5–16.5 fetal livers were disaggregated in 2% FBS-RPMI by multiple passages through a 26-gauge needle and filtered through a 70 μm strainer (BD Falcon). E1–positive cells were enriched by positive selection using streptavidin-coupled magnetic beads and an autoMACS cell separator (Miltenyi Biotec, Auburn, CA). Depletion of Lineage (Lin)− cells was done by incubation with purified Lin antibodies (see below) followed by magnetic beads (Dynal, Life Sciences, Amersham, UK).
Technologies) as per manufacturer instructions. HSCs were stained with unconjugated Lin antibodies Gr-1 (1:800), Mac1 (1:100), B220 (1:800), CD3 (1:100), CD4 (1:500), CD8a (1:100), Ter-119 (1:100), CD45 (1:100), Tert-1 (1:100), obtained from the UCSF-HybridoLine Core Facility), goat anti-rat-PE-Cy5 (Invitrogen, A10691; 1:400), c-Kit-APC-eFlour780 (eBioscience, 47-1171-82; 1:800), Sca-1-PE (BioLegend, 108120; 1:400), Filk2-Bio (eBioscience, 13-1351-82; 1:100), CD48-Alexa Fluor 647 (BioLeg-


don excluded. At least 20,000 events were collected from the single alive population.

Blood sampling

Star, Ashland, OR). Blood was sampled on a FACSAriaII (BD, San Jose, CA) and data was analyzed with FlowJo 9.7.5 (Tree

labelled with surface markers Ter119 and CD71, fixed, permeabilized and labelled with the following cytokines (all from PeproTech): IL-3 (10 ng ml−1), IL-11 (25 ng ml−1), Flt-3L (25 ng ml−1), Tpo (25 ng ml−1) and Epo (4 U ml−1) (culture media). When indicated, 50 ng ml−1 aphidicolin (Sigma, A4487) was added to the culture media. Colonies (triplicates) were counted on day 7. All cultures were maintained at 37 °C in a 5% CO2 water jacket incubator (Thermo Scientific).

Statistical methods. In column graphs, data are expressed as mean ± s.d. Statistical analyses were done using Fisher’s test. When the data are presented in scatter dot plots, the bar corresponds to the median value. For the analyses of FR, IOD and fork symmetry parameters in stretched DNA fibers and immunofluorescence data from acquired in the high-throughput Opera system, data distribution was normally not Gaussian, and differences between samples were assessed with the non-parametric Mann-Whitney rank-sum test. In the analysis of apoptosis and cell death in EB populations, the mean values derived from 3 independent experiments were compared by One-Way Anova and further Bonferroni post-test. Statistical analysis was performed in Prism v4.0 (GraphPad Software).

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