LETTERS

Cdk1 is sufficient to drive the mammalian cell cycle

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Unicellular organisms such as yeasts require a single cyclin-dependent kinase, Cdk1, to drive cell division[1]. In contrast, mammalian cells are thought to require the sequential activation of at least four different cyclin-dependent kinases, Cdk2, Cdk3, Cdk4 and Cdk6, to drive cells through interphase, as well as Cdk1 to proceed through mitosis[2]. This model has been challenged by recent genetic evidence that mice survive in the absence of individual interphase Cdks[3–8]. Moreover, most mouse cell types proliferate in the absence of two or even three interphase Cdks[8–10]. Similar results have been obtained on ablation of some of the activating subunits of Cdks, such as the D-type and E-type cyclins[11–14]. Here we show that mouse embryos lacking all interphase Cdks (Cdk2, Cdk3, Cdk4 and Cdk6) undergo organogenesis and develop to midgestation. In these embryos, Cdk1 binds to all cyclins, resulting in the phosphorylation of the retinoblastoma protein pRb and the expression of genes that are regulated by E2F transcription factors. Mouse embryonic fibroblasts derived from these embryos proliferate *in vitro*, albeit with an extended cell cycle due to inefficient inactivation of Rb proteins. However, they become immortal on continuous passage. We also report that embryos fail to develop to the morula and blastocyst stages in the absence of Cdk1. These results indicate that Cdk1 is the only essential cell cycle Cdk. Moreover, they show that in the absence of interphase Cdks, Cdk1 can execute all the events that are required to drive cell division.

When $Cdk4^{+/-}$;$Cdk6^{-/-}$;$Cdk2^{+/-}$ mice are crossed, 25% of the conceptuses should be triple knockout (TKO) $Cdk4^{-/-}$;$Cdk6^{-/-}$;$Cdk2^{-/-}$, as *Cdk4* and *Cdk2* are genetically linked[9,10]. We obtained TKO embryos at the expected mendelian ratios from such crosses (Supplementary Table 1). These embryos underwent morphogenesis and organogenesis and developed until embryonic day 12.5 (E12.5) (Fig. 1a). Cells proliferated in most tissues, as measured by staining for the proliferative marker Ki67 (Fig. 1a, Supplementary Fig. 1a). However, TKO embryos were not normal and began to die at E13.5, with all dying by E15.5. The livers of live TKO embryos at E12.5–E13.5 showed a threefold reduction in cellularity, a decrease that is significantly higher than expected from their smaller size (25–40%). The levels of haematopoietic stem cells, granulocyte-macrophage progenitors and megakaryocyte-erythroid progenitors were consistent with the overall reduction in liver cellularity. Only common myeloid progenitors showed a greater (eightfold) reduction (Supplementary Fig. 1b). More importantly, livers from TKO embryos, unlike those from $Cdk4^{-/-}$;$Cdk6^{-/-}$embryos, undergo substantial apoptosis at E13.5–E14.5, as determined by active caspase 3 immunoreactivity (Supplementary Fig. 1b). The hearts of TKO embryos showed thinner ventricular walls than those of wild-type controls owing to a decrease in the number of proliferating cardiomyocytes (data not shown). This phenotype was indistinguishable from that of $Cdk4^{-/-}$;$Cdk2^{-/-}$ embryos, which could complete embryonic development[10]. Thus, although we cannot rule out the possibility that the heart phenotype contributed to embryonic death, the severity of the haematopoietic defects makes them more likely to account for the failure of TKO embryos to develop beyond midgestation.

The loss of interphase Cdks did not have a significant effect on the levels of expression of other cell-cycle regulators. The overall levels of Cdk1, Cdk7 and Cdk9 appeared to be unchanged (Fig. 1b). There were no significant variations in the expression of the cyclins tested (D1, D2, E1, A2 and B1). Among Cip/Kip inhibitors, only cyclin-dependent kinase inhibitor 1B (Cdkn1b, also known as $p27^{Kip1}$) showed decreased protein levels in TKO embryos (Fig. 1b), probably owing to increased protein instability, as the levels of *Cdkn1b* messenger RNA were not affected (Supplementary Fig. 2). pRb was phosphorylated in TKO embryos, including at those residues (Ser 608, Ser 780 and Ser 807/811) that were previously thought to be specific targets of interphase Cdks[15] (Fig. 1c).

To understand the molecular mechanisms that drive cell division in the absence of interphase Cdks, we investigated whether Cdk1 could interact with G1/S cyclins. As shown in Fig. 1d, Cdk1 interacts with cyclin D1 and cyclin D2 in lysates derived from embryos lacking Cdk4. Likewise, Cdk1 binds to cyclin E in $Cdk2^{-/-}$ and $Cdk4^{-/-}$;$Cdk2^{-/-}$ embryos, as previously reported[10,16]. Moreover, cyclin D and E immunoprecipitates from TKO embryos could phosphorylate recombinant pRb (Fig. 1e), possibly owing to their ability to form complexes with Cdk1.

The proliferation of primary mouse embryonic fibroblasts (MEFs) in culture was partially compromised in the absence of interphase Cdks. However, most TKO MEF cultures (11 of 12) became immortal upon continuous passage (Supplementary Fig. 3a, b). These TKO MEFs, as well as control cultures that expressed at least one interphase Cdk, maintained the expression of cyclin-dependent kinase inhibitor 2A (Cdkn2a, also known as $p16^{INK4a}$) and a functional P53 response (data not shown). Immortal TKO cultures, unlike those expressing at least one interphase Cdk, retained their extended doubling time. To understand the molecular basis of the slower cell cycle of TKO MEFs, we infected them with a retrovirus that expressed a fragment of the simian virus 40 (SV40) large T antigen (T_{121}) that is known to inactivate the three members of the Rb family[17]. Inactivation of all pocket proteins made the proliferation rates of TKO MEFs indistinguishable from those of wild-type cells (Supplementary Fig. 3c). These observations indicate that the slower cell cycles of TKO MEFs are a consequence of incomplete inactivation of Rb proteins. We obtained similar results from TKO MEFs derived from immortal $Cdk4^{-/-}$;$Cdk6^{-/-}$;$Cdk2^{lox/lox}$ cells after infection with a retrovirus expressing the Cre recombinase (Supplementary Fig. 3d), ruling out the idea that the proliferation of TKO MEFs was a consequence of embryonic plasticity.

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TKO MEFs exited quiescence when stimulated with serum, albeit with significantly delayed (6–8 h) kinetics (Fig. 2a). However, most primary and immortal TKO MEFs entered the cell cycle (Fig. 2b) and executed the molecular steps that are known to be required to proceed into S phase, including pRb phosphorylation, E2F-dependent induction of cyclin A2 expression and degradation of the Cdk inhibitor Cdkn1b (Fig. 2c). In addition, Cdk1 formed complexes with cyclin D1 as early as 12 h after serum stimulation. These complexes were observed only at later time points (beyond 24 h) in control MEFs (Fig. 2d). Thus, it is tempting to speculate that Cdk1–cyclin D complexes might be functionally relevant during mitotic exit and/or early G1 phase, a period when other cyclins are being actively targeted by the anaphase-promoting complex/cyclosome[18].

Next, we investigated whether Cdk1–cyclin complexes were responsible for allowing TKO cells to enter S phase. We infected TKO MEFs with lentiviral vectors expressing either control or short hairpin RNAs (shRNAs) specific for *Cdc2a*, the locus that encodes Cdk1. Knockdown of Cdk1 had no effect on the ability of cells expressing Cdk4 and Cdk2 to exit G0 and enter S phase (Fig. 2e). However, Cdk1 depletion completely abrogated S phase entry in TKO MEFs, an effect that correlated with the inhibition of pRb phosphorylation (Fig. 2e). These observations indicate that, in the absence of interphase Cdks, Cdk1 can be activated by D-type and/or E-type cyclins to phosphorylate pRb and bring cells out of quiescence.

Finally, we investigated whether Cdk1 was essential for cell division. To this end, we generated mutant mice that were heterozygous for *Cdc2a* from two independent embryonic stem cells that carried β-geo cassette insertions within this locus[19,20] (Fig. 3a). These integration events generate a Cdk1–β-geo fusion protein that contains only the 12 amino-terminal residues of Cdk1 (Fig. 3b). Heterozygous $Cdc2a^{+/mut1}$ cells express about 50% as much Cdk1 as is present in wild-type cells (Fig. 3c). Crosses between heterozygous $Cdc2a^{+/mut1}$ or $Cdc2a^{+/mut2}$ mice did not yield homozygous *Cdc2a* mutant animals nor midgestation embryos (E10.5–E13.5) (Fig. 3d). Likewise, we could not identify E2.5 (morula stage) or E1.5 (2–4-cell) $Cdc2a^{mut1/mut1}$ or $Cdc2a^{mut2/mut2}$ embryos. Analysis of 23 morulae that were allowed to grow in culture for 5–6 days using nested primers also failed to identify homozygous $Cdc2a^{mut1/mut1}$ embryos (Fig. 3d). Moreover, the percentage of wild-type and $Cdc2a^{+/mut1}$ embryos were those expected if homozygous embryos were not viable. Finally, crosses between wild-type and $Cdc2a^{+/mut1}$ male and female mice yielded the expected percentage of $Cdc2a^{+/mut1}$ offspring (data not shown), indicating that

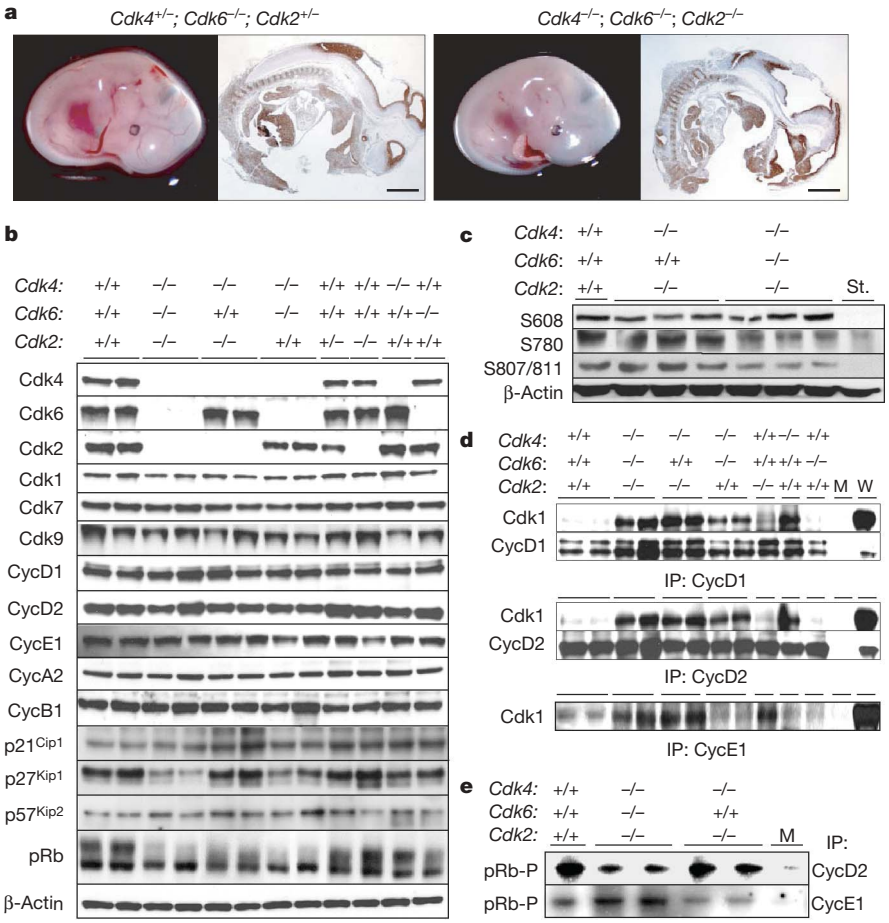


Figure 1 | Characterization of embryos lacking all interphase Cdks. **a**, Embryos lacking all interphase Cdks undergo organogenesis and develop until midgestation. Left, $Cdk4^{+/-}$;$Cdk6^{-/-}$;$Cdk2^{+/-}$ E12.5 embryos. Right, $Cdk4^{-/-}$;$Cdk6^{-/-}$;$Cdk2^{-/-}$ E12.5 embryos. In both cases, the left image depicts a picture of the whole embryo and the right image depicts a Ki67-immunostained sagittal section (×12). Scale bar, 1mm. **b**, Expression of cell cycle regulators in independent E12.5 embryos of the indicated genotype. Results from TKO embryos are shown next to results from wild-type embryos (left column). Protein extracts were analysed by immunoblotting with antibodies elicited against the indicated proteins (see Methods). Expression of β-actin served as loading control. **c**, Analysis of pRb phosphorylation in wild-type, $Cdk4^{-/-}$;$Cdk6^{+/+}$;$Cdk2^{-/-}$ and $Cdk4^{-/-}$;$Cdk6^{-/-}$;$Cdk2^{-/-}$ E12.5 embryos using antibodies specific for phosphorylated residues S608, S780 and S807/811. St.: pRb phosphorylation at these residues in TKO serum-starved cells. **d**, Extracts from E12.5 embryos of the indicated genotype were immunoprecipitated with antibodies against cyclin D1 (top), cyclin D2 (middle) and cyclin E1 (bottom) and analysed by immunoblotting using antiserum against Cdk1, cyclin D1 and cyclin D2 as indicated. Results from two independent embryos are shown for wild-type, TKO and double mutant embryos. M, mock immunoprecipitate; W, whole cell extract at a 1:10 dilution before immunoprecipitation. **e**, *In vitro* kinase activity associated with cyclin D2 (top) and cyclin E1 (bottom) immunoprecipitates using pRb as substrate. M, mock immunoprecipitate.

Cdc2a^{mut1} oocytes and spermatozoa are viable. These observations indicate that Cdk1 is essential for the early stages of embryonic development.

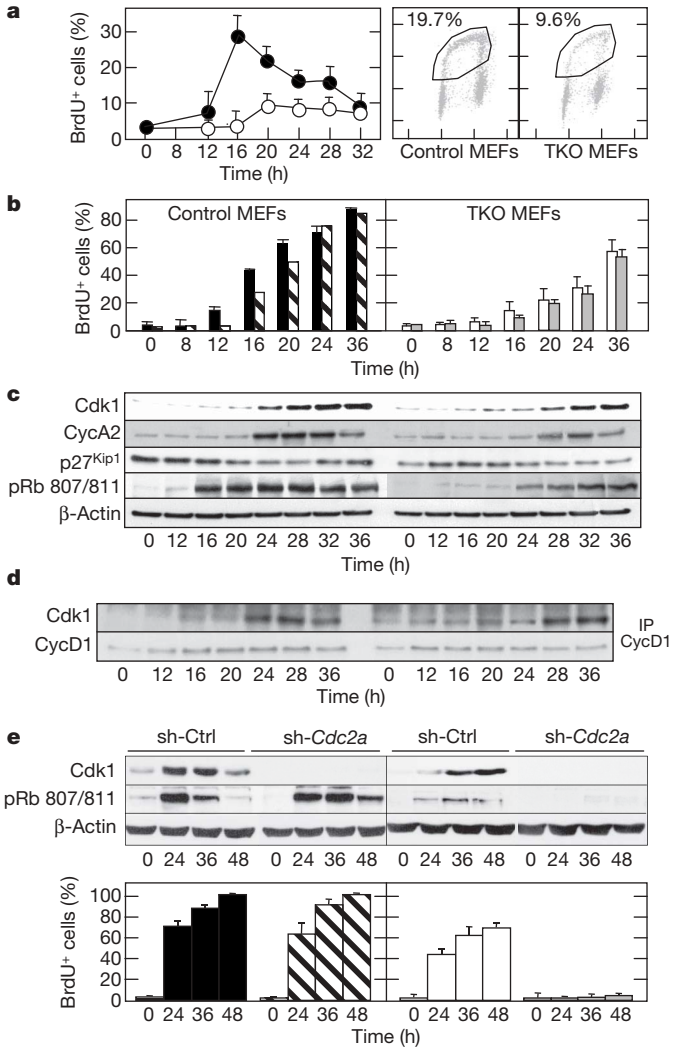
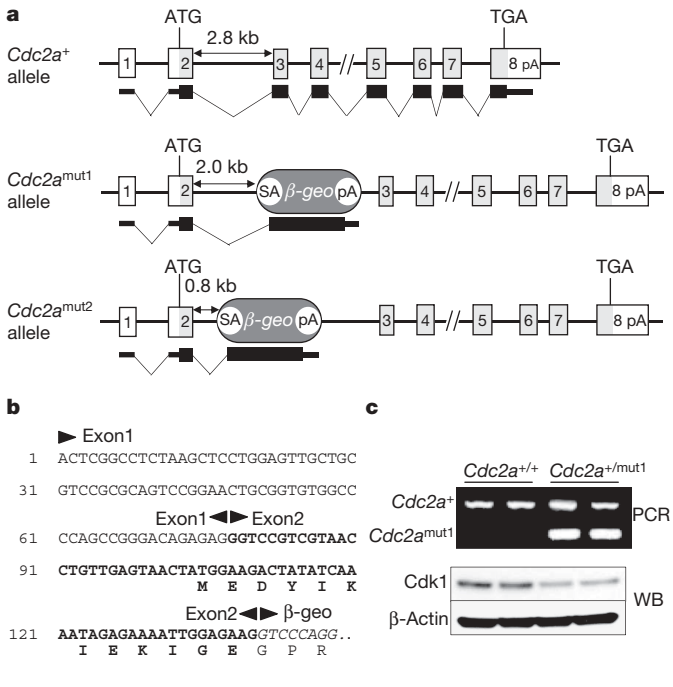


Figure 2 | Mitogenic response of TKO MEFs. a, Left, percentage of quiescent *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$;*Cdk2*$^{+/+}$ (filled circles) and *Cdk4*$^{-/-}$;*Cdk6*$^{-/-}$;*Cdk2*$^{-/-}$ (open circles) primary MEFs in S phase after serum stimulation following 2-h BrdU pulses. Right, representative analysis of BrdU incorporation 20 h after serum stimulation of the above MEFs. Numbers indicate the percentage of BrdU^{+} cells in S phase. **b**, Percentage of quiescent primary (solid bars) and immortal (hatched bars) *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$;*Cdk2*$^{+/+}$ control MEFs and primary (empty bars) and immortal (grey bars) *Cdk4*$^{-/-}$;*Cdk6*$^{-/-}$;*Cdk2*$^{-/-}$ TKO MEFs entering S phase upon addition of 10% FBS in the continuous presence of BrdU at the indicated times after serum stimulation. Data shown as mean ± s.d., $n = 3$. **c**, Immunoblot analysis of Cdk1, cyclin A2 and p27^{Kip1} expression and phosphorylation of S807/811 pRb residues in immortal MEFs at the indicated times after the addition of serum. Expression of β-actin serves as a loading control. **d**, Formation of Cdk1–cyclin D1 complexes during G0 exit in immortal MEFs shown in **b**. Extracts were immunoprecipitated using antibodies against cyclin D1 and analysed by immunoblotting using antisera against Cdk1. Immunoprecipitation of cyclin D1 is shown as a control. **e**, Knock-down of *Cdk1* abolishes G0 exit in TKO but not in control MEFs. Upper, expression of Cdk1 and phosphorylation of S807/811 pRb residues in *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$;*Cdk2*$^{+/+}$ (left) and *Cdk4*$^{-/-}$;*Cdk6*$^{-/-}$;*Cdk2*$^{-/-}$ (right) MEFs infected with lentiviruses expressing control (sh-Ctrl) or *Cdc2a*-specific (sh-*Cdc2a*) shRNAs, at the indicated times after serum stimulation. β-actin serves as a loading control. Lower, percentage of quiescent *Cdk4*$^{+/+}$;*Cdk6*$^{-/-}$;*Cdk2*$^{+/+}$ (left) and *Cdk4*$^{-/-}$;*Cdk6*$^{-/-}$;*Cdk2*$^{-/-}$ (right) MEFs entering S phase upon addition of 10% FBS in the continuous presence of BrdU infected with lentiviruses expressing a control shRNA (solid and empty bars, respectively) or an shRNA specific for *Cdc2a* (hatched and grey bars, respectively). Data shown as mean ± s.d., $n = 3$.

Eukaryotic evolution has endowed multicellular organisms with an increasing number of loci encoding Cdks, reaching at least 12 (and potentially 20) in mammals[2]. Unlike in yeasts, where Cdk1 is sufficient to drive the cell cycle, in mammals at least four additional Cdks (Cdk2, Cdk3, Cdk4 and Cdk6) have been proposed to drive the various phases of their cell cycle in sequence[1,2]. Recent genetic evidence has challenged some of the concepts behind this model. For instance, most laboratory strains of mice are deficient in Cdk3 owing to a naturally occurring mutation[3]. Moreover, Cdk4 and Cdk6 are not essential for resting cells to exit quiescence[8]. Likewise, cells lacking Cdk2, a kinase that was previously thought to be essential for driving the S phase, divide normally[6,7]. Finally, MEFs lacking Cdk4 and Cdk2 proliferate and become inmortal[10]. *In vivo*, single and double Cdk mutant mice show proliferative defects, but only in specific cell types (Fig. 4). Moreover, as illustrated here, embryos



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Stage	*n*	Genotype		
		Cdc2a$^{+/+}$	*Cdc2a*$^{+/mut1}$	*Cdc2a*$^{mut1/mut1}$
P21	17	5 (29%)	12 (71%)	0
E10.5–E13.5	51	21 (41%)	30 (59%)	0
E2.5	39	14 (36%)	25 (64%)	0
E1.5	18	5 (28%)	13 (72%)	0

Figure 3 | Cdk1 is essential for early embryonic development. a, Schematic representation of wild type *Cdc2a*$^{+}$ (encoding Cdk1), *Cdc2a*^{mut1} and *Cdc2a*^{mut2} alleles. The mutant alleles contain an inserted β-geo trap cassette (dark grey) at different positions within intron 2. *Cdc2a* exons are indicated by numbered boxes. Non-coding (open boxes) and coding (light grey boxes) sequences are indicated. Thin (non-coding) and thick (coding sequences) lines represent the expected mRNAs derived from each allele. ATG, initiation codon; TGA, terminator codon; SA, splicing acceptor; *β-geo*, bacterial β-galactosidase/neomycin-resistance gene; pA, polyadenylation signal. **b**, Nucleotide sequence of the 5′RACE product derived from *Cdc2a*$^{+/mut1}$ embryonic stem cells. The boundaries of exon 1, exon 2 (bold) and *β-geo* (italics) are indicated. The amino-acid residues of the predicted Cdk1–β-geo chimaeric protein are also indicated. Bold residues correspond to those of Cdk1. Residues in italics are those encoded by β-geo sequences. An additional glycine residue is expected to be generated during splicing. **c**, Top, genotyping of *Cdc2a*$^{+/+}$ and *Cdc2a*$^{+/mut1}$ mice by PCR amplification of *Cdc2a*$^{+}$ and *Cdc2a*^{mut1} alleles. Bottom, western blot (WB) analysis of Cdk1 protein levels in primary *Cdc2a*$^{+/mut1}$ and wild-type MEFs. **d**, Number of mice (P21) and embryos (E10.5–E13.5, E2.5 and E1.5) of the indicated genotype derived from crosses between *Cdc2a*$^{+/mut1}$ mice. Percentage of *Cdc2a*$^{+/+}$ and *Cdc2a*$^{+/mut1}$ mice and embryos is indicated in parentheses.

lacking all interphase Cdks undergo organogenesis and develop to midgestation, indicating that Cdk1 can drive the essential cell cycle (Fig. 4). Indeed, only mice lacking Cdk1 (this study), cyclin A2 and possibly cyclin B1 have impaired cell division during the very early stages of embryonic development[21,22], which indicates that Cdk1 kinase activity cannot be compensated by interphase Cdks.

These observations suggest that Cdks display a significant degree of plasticity. As shown here, Cdk1 can interact with all cyclins, a property that was previously thought to be unique to unicellular organisms. More importantly, these Cdk1 complexes are biochemically active. But these interactions, at least *in vitro*, are only observed in the absence of their 'cognate' Cdk, mainly Cdk4 and Cdk2 (Fig. 1d). Thus, if these preferred Cdk–cyclin interactions also occur *in vivo*, Cdk1 would only drive interphase in the absence of the other Cdks (Fig. 4). A possible resolution to this issue might be provided by strains of mice that express kinase-inactive Cdks rather than null alleles.

Genetic analysis does not explain how Cdk1 can compensate for the absence of interphase Cdks during embryonic development, the most active period of cell division, but not in certain specialized cell types. The most plausible explanation is that interphase Cdks, but not Cdk1, phosphorylate substrates that are unique to those cell types in which they are essential. It is also possible that in these specialized cells, only interphase Cdks can generate the necessary levels of kinase activity to drive cell division. This scenario might explain why TKO MEFs achieve normal levels of cell proliferation only in the presence of SV40 T_{121}, which inactivates the Rb family of proteins (Supplementary Fig. 3c). A precise understanding of how each Cdk contributes to drive cell division should help us to develop appropriate therapeutic strategies to treat cancer as well as other diseases caused by aberrant cell proliferation.

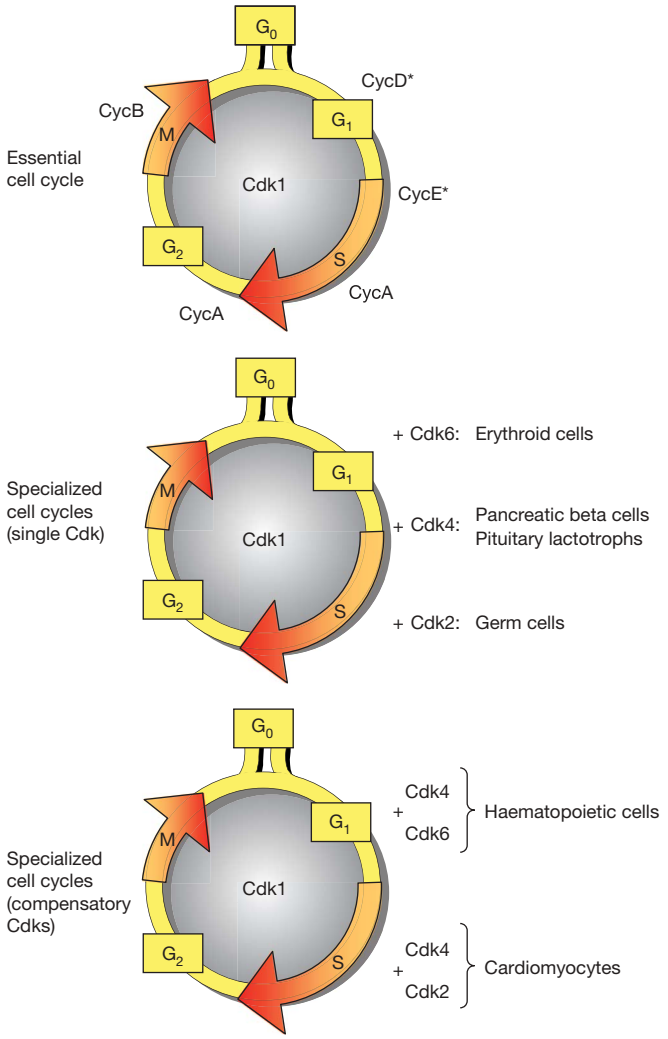

Figure 4 | Summary of genetic results obtained by ablation of loci encoding cell-cycle Cdks in mice. The diagram depicting the 'essential cell cycle' (top) is based on our results. The asterisks indicate that D-type or E-type cyclins are not essential for cell proliferation[11–14]. It is not known whether cells expressing Cdk1 in the absence of D-type and/or E-type cyclins would proliferate. The cartoons depicting 'specialized cell cycles' summarize previous work illustrating which interphase Cdk (middle) or Cdks (bottom) are essential for proliferation of the indicated cell types. The location of Cdks within the phases of the cell cycle is arbitrary. Cyclins have been omitted for clarity. References can be found in the text.

METHODS SUMMARY

Gene targeting and mouse strains. *Cdk4*$^{-/-}$;*Cdk2*$^{-/-}$, *Cdk6*$^{-/-}$ and *Cdk2*^{lox} mice have been described[6,8,10]. *Cdc2a*$^{+/mut1}$ embryonic stem cells were obtained in a gene trap screen[19] and *Cdc2a*$^{+/mut2}$ embryonic stem cells were obtained from the German Gene Trap Consortium[20].

Cell culture assays. MEFs were isolated from E12.5 embryos. All cell culture experiments were carried out in fibronectin-coated plates (Becton Dickinson) and 3% oxygen. MEFs were propagated according to standard 3T3 protocols. For proliferation assays, we plated 5×10^4 cells on six-well plates in duplicate as described[23]. To analyse S phase entry, we deprived MEFs of serum for 72 h in DMEM plus 0.1% FBS and re-stimulated them with 10% FBS. Cells were either pulsed (2 h) or continuously labelled with 50 μM bromodeoxyuridine, harvested at the indicated times and stained with anti-BrdU fluorescent antibodies. MEFs were infected with retroviral vectors as described[23]. Knockdown of *Cdc2a* was achieved with lentiviral shRNA delivery. After infection, cells were selected with 2 μg ml^{-1} puromycin for 72 h in DMEM plus 0.1% FBS and re-stimulated with 10% FBS.

Analysis of haematopoietic precursors. Livers were collected from E12.5–E13.5 embryos and disrupted to form single-cell suspensions. Cells were immunostained and haematopoietic progenitors identified as described[8].

Protein analysis. Protein lysates from either whole embryos or cells were prepared and used for immunoblotting as described[23]. For kinase assays, 300 μg of whole-cell extract was immunoprecipitated and the resulting immunoprecipitates were incubated with 1 μg of mouse pRb protein fragment or histone H1 as substrates[23]. Cdk–cyclin complexes were analysed by immunoprecipitation of 300 μg of whole-embryo extracts.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.B. supervised the entire project. M.B., D.S. and M.M. conceived and designed the experiments, and wrote the manuscript with comments from co-authors. C.B. generated the mouse strains and carried out most cell culture experiments and part of the protein analysis. A.C. was responsible for most protein analysis and part of the cell culture experiments. S.H. and C.T. characterized *Cdc2a* mutant mice. P.D. was responsible for histopathological analysis. J.F.C. and K.N. generated one of the *Cdc2a* mutant embryonic stem cell clones.

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METHODS

Gene targeting and mouse strains. *Cdk4*$^{-/-}$;*Cdk2*$^{-/-}$, *Cdk6*$^{-/-}$ and *Cdk2*^{lox} mice have been described[6,8–10]. *Cdc2a*$^{+/mut1}$ embryonic stem (ES) cells were obtained in a gene trap screen[19]. Neo^{R} colonies were screened for β-galactosidase activity with 5-bromo-4-chloro-3 indoyl β-D-galactosidase (X-Gal) and analysed by immunofluorescence using antibodies specific for β-gal. To characterize the trapped allele, we prepared total RNA using Bio/RNA-X-cell (Bio/Gene Limited). 5′ RACE products were generated and directly sequenced using Big DyeTM terminator kit using a –40 lacZ primer (USB/Amersham) on an ABI prism 377 DNA sequencer. *Cdc2a*$^{+/mut2}$ ES cells were obtained from the German Gene Trap Consortium (clone P009F08; http://www.genetrap.org). The sequence of the oligonucleotides used for genotyping is available upon request. Mice used in this study have been maintained in a mixed 129/SvJ x C57BL/6J background according to the Guide of the Care and Use of Laboratory Animals (ILAR 1996) and in accordance with the Spanish Animal Protection Law (RD1201/2005) and the European Directive (86/609/CEE) established by the European Union to regulate animal care standards.

Histopathology and immunohistochemistry. Embryos were fixed in 10%-buffered formalin (Sigma) and embedded in paraffin. Three- or five-micrometer-thick sections were stained with haematoxylin and eosin. For proliferation studies, tissue sections were stained with Ki67-specific antibodies (MIB-1; Dako). Apoptotic cells were detected using anti-active Caspase 3 antibodies (R&D Systems).

Cell culture assays. MEFs were isolated from E12.5 embryos and cultured in fibronectin-coated plates (Becton Dickinson) and 3% oxygen in the presence of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). MEFs were propagated according to standard 3T3 protocols. For proliferation assays, 5×10^4 cells were plated on six-well plates in duplicate as described[23]. To analyse S phase entry, we deprived MEFs (10^6 cells per 10-cm dish) of serum for 72 h in DMEM plus 0.1% FBS and re-stimulated with 10% FBS. Cells were either pulsed (2 h) or continuously labelled with 50 μM bromodeoxyuridine (BrdU; Sigma), harvested at the indicated times and stained with anti-BrdU fluorescent antibodies (Becton Dickinson). MEFs were infected with retroviral vectors as described[23]. Knockdown of *Cdc2a* was mediated with lentiviral Mission–shRNA plasmids (SHGLY-NM_007659, Sigma) according to the manufacturer's instructions. After infection, cells were selected with 2 μg ml^{-1} puromycin for 72 h in DMEM plus 0.1% FBS and re-stimulated with 10% FBS.

Analysis of haematopoietic precursors. Livers were collected from E12.5–E13.5 embryos and disrupted to form single-cell suspensions. Cells were immunostained and haematopoietic progenitors identified as described[8]. Haematopoietic stem cells were gated as Lin$^-$ IL7Rα$^-$ c-Kit$^+$ Sca1$^+$; common myeloid progenitors as Lin$^-$ IL7Rα$^-$ c-Kit$^+$ Sca1$^-$ FcγR^{low} CD34$^+$; granulocyte-macrophage progenitors as Lin$^-$ IL7Rα$^-$ c-Kit$^+$ Sca1$^-$ FcγR^{hi} CD34$^+$; and megakaryocyte-erythroid progenitors as Lin$^-$ IL7Rα$^-$ c-Kit$^+$ Sca1$^-$ FcγR^{low} CD34$^-$. Their relative numbers were quantified using a FACSAria (Becton Dickinson) cytometer.

Protein analysis. Protein lysates were prepared and used for immunoblotting as described[23]. Antibodies against the following proteins were used: Cdk1 (17; Santa Cruz), Cdk4 (C22; Santa Cruz), Cdk2 and Cdk6 (our own rabbit polyclonal antibodies), Cdk7 (C22; Santa Cruz), Cdk9 (C22; Santa Cruz), Cyclin A2 (H432; Santa Cruz), Cyclin B1 (Santa Cruz), Cyclin D1 (DCS6 and AB4; Neo Markers), Cyclin D2 (Santa Cruz), Cyclin E1 (M20; Santa Cruz), p21^{Cip1} (C19; Santa Cruz), p27^{Kip1} (Transduction Laboratories), p57^{Kip2} (C20; Santa Cruz), β-Actin (AC15, Sigma) and pRb (BD Pharmingen). pRb phosphospecific antibodies to phosphorylated residues S608 (#2181), S780 (#9307) and S807/811 (#9308) were from Cell Signaling. As secondary antibodies, we used peroxidase-conjugated IgG (Dako), followed by chemiluminescence detection (ECL; Amhersam). For kinase assays, 300 μg of whole cell extract was immunoprecipitated with the indicated antibodies and the resulting immunoprecipitates incubated with 1 μg of mouse pRb protein fragment (amino-acid residues 769–921; Santa Cruz) or histone H1 (calf thymus, Roche) as substrates[23].

***Cdkn1b* expression analysis by real-time quantitative PCR.** Total RNA was isolated from cells using RNAeasy (Quiagen) and digested with DNaseI to remove contaminating DNA. cDNA was prepared from 2 μg of total RNA using Superscript II Reverse transcriptase kit (Invitrogen). Real-time PCR was performed on several cDNA dilutions plus 1x SYBR Green PCR Master Mix (Applied Biosystems) and 50 nM of *Cdkn1b* primers (sequence available upon request). Reactions were carried out using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) for 40 cycles (95 °C for 15 s and 60 °C for 1 min) after an initial 10-min incubation at 95 °C. Variation of *Cdkn1b* expression was calculated using the ΔΔCt method[24] with *β-actin* mRNA as an internal control.

24. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25**, 402–408 (2001).