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Genetic substitution of Cdk1 by Cdk2 leads to embryonic lethality and loss of meiotic function of Cdk2

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It was believed that Cdk2-cyclin E complexes are essential to drive cells through the G1–S phase transition. However, it was discovered recently that the mitotic kinase Cdk1 (Cdc2a) compensates for the loss of Cdk2. In the present study, we tested whether Cdk2 can compensate for the loss of Cdk1. We generated a knockin mouse in which the *Cdk2* cDNA was knocked into the *Cdk1* locus (*Cdk1*^{Cdk2KI}). Substitution of both copies of *Cdk1* by *Cdk2* led to early embryonic lethality, even though *Cdk2* was expressed from the *Cdk1* locus. In addition, we generated *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice in which one copy of *Cdk2* and one copy of *Cdk1* were expressed from the *Cdk1* locus and the *Cdk2* gene was deleted from the endogenous *Cdk2* locus. We found that both male and female *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice were sterile, similar to *Cdk2*$^{-/-}$ mice, even though they expressed the Cdk2 protein from the *Cdk1* locus in testes. The translocational and cell cycle properties of knockin Cdk2 in *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ cells were comparable to those of endogenous Cdk2, but we detected premature transcriptional activation of *Cdk1* during liver regeneration in the absence of Cdk2. This study provides evidence of the molecular differences between Cdk2 and Cdk1 and highlights that the timing of transcriptional activation and the genetic locus play important roles in determining the function of Cdk proteins in vivo.

KEY WORDS: Cell cycle regulation, Cyclin, Cyclin-dependent kinase (Cdk), Meiosis, Mouse genetics

INTRODUCTION

Cyclin-dependent kinases (Cdks) together with cyclins constitute the central components of the cell cycle machinery (Morgan, 1997). In eukaryotic cells, several Cdk-cyclin complexes, including Cdk2-cyclin E, Cdk1-cyclin B, Cdk4-cyclin D, Cdk6-cyclin D and Cdk2-cyclin A, drive cell cycle progression, and it was believed that their functions are confined to specific stages of the cell cycle (Morgan, 1997). For example, Cdk4 and Cdk6 are thought to be involved in early G1, whereas Cdk2 is essential to complete G1 and initiate S-phase. Cdk4 and Cdk6 form active complexes with D-type cyclins to initiate the cell division cycle by phosphorylating the Retinoblastoma protein (Rb). Thereafter, in late G1 phase, the activation of Cdk2 by cyclin E further phosphorylates Rb and drives cells through the G1–S restriction point. Later, Cdk2 complexes with cyclin A and is essential for S-phase progression (for a review, see Mittnacht, 1998; Weinberg, 1995). Recently, it was reported that Cdk2 can also interact with the mitotic cyclin, cyclin B, but the exact function of this complex is not known (Aleem et al., 2005). In addition, it has been reported that Cdk2 is present predominantly in the nucleus throughout the cell cycle (Moore et al., 1999; Pines and Hunter, 1991; Satyanarayana et al., 2008). Cdk1 (Cdc2a – Mouse Genome Informatics), in association with cyclin B, is essential to control entry into and exit from mitosis; Cdk1 is present mainly in the cytoplasm and translocates to the nucleus only during mitosis after complexing with cyclin B (Dunphy et al., 1988; Izumi and Maller, 1993; Pan et al., 1993; Riabowol et al., 1989).

In contrast to mammalian cells, in budding yeast a single Cdk, the transcriptional product of the *CDC28* gene, regulates diverse cell cycle transitions by associating with multiple stage-specific cyclins (Nasmyth, 1993; Reed et al., 1982). On the basis of the concepts derived from the yeast cell cycle, it was hypothesized that the functions of the multiple Cdks in eukaryotic cells are redundant and one or two Cdks might be sufficient to drive cells through the different phases of the cell cycle. In support of this, recent studies have demonstrated that *Cdk2*, *Cdk4* and *Cdk6* single-knockout mice are viable, do not show any severe phenotypes and display minor defects in cell cycle properties, indicating functional redundancy between the different Cdks (Berthet et al., 2003; Malumbres et al., 2004; Ortega et al., 2003). Notably, Cdk1, which was originally identified as an essential mitosis-promoting kinase, can compensate for the loss of Cdk2 by complexing with cyclin E to drive cells through the G1–S transition, even though Cdk1 is only ~65% identical to Cdk2 (Aleem et al., 2005). In addition, a recent study has demonstrated that Cdk1 alone is sufficient to drive the eukaryotic cell cycle in early embryogenesis and in mouse embryonic fibroblasts (MEFs) (Santamaria et al., 2007). However, at the whole-organism level, the compensation of Cdk2 function by Cdk1 appears to be only partial, as *Cdk2* knockout males and females are sterile, displaying dysfunctional and atrophic testes and ovaries (Berthet et al., 2003; Ortega et al., 2003). This indicated that Cdk2 is essential for meiosis and that Cdk1 cannot functionally compensate for the loss of Cdk2. In this context, it is of interest to explore whether there are any possible ways in which Cdk2 might compensate for the loss of Cdk1. Deletion of *Cdk1* or a gene-trap mutation in the *Cdk1* gene leads to early embryonic lethality (our unpublished results) (Santamaria et al., 2007), indicating that Cdk1 is essential for the survival of mice. This implies that Cdk2 cannot compensate for the loss of Cdk1 when expressed from its own locus. The inability of Cdk2 to take over the function of Cdk1 could be attributed to: (1) intrinsic differences between the Cdk1 and Cdk2 proteins, such as substrate specificity or

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interaction with binding partners; (2) differences in the timing of expression of Cdk1 and Cdk2 during the different phases of cell cycle; and/or (3) differences in their sub-cellular localization. It is of interest to explore whether Cdk2 acquires some of the properties of Cdk1 when Cdk2 is expressed directly from the *Cdk1* locus in vivo, and whether it would be able to compensate for the loss of Cdk1. This hypothesis is derived from recent findings that genetic replacement of cyclin D1 by cyclin E can rescue the phenotypes of cyclin D1 knockout mice (Geng et al., 1999). Similarly, it has been shown that cyclin D2 rescues the loss of cyclin D1 when expressed from the D1 locus (Carthon et al., 2005). Furthermore, H-Ras (Hras1) can substitute for K-Ras (Kras) and supports normal embryonic development (Potenza et al., 2005). These studies provide evidence that the timing of expression and the genetic locus play important roles in determining the functions of a protein. By genetically replacing Cdk1 with Cdk2, it is possible to study whether Cdk2 can rescue the loss of Cdk1 in vivo. At the same time, it is of interest to determine whether Cdk2 can retain its own functions when expressed from the *Cdk1* locus. In this context, it is also important to determine how efficiently Cdk2 performs its own mitotic cell cycle and meiotic functions in germ cells when expressed from the *Cdk1* locus, as Cdk1 cannot functionally rescue the meiotic functions of Cdk2 in *Cdk2*$^{-/-}$ mice.

To better understand the importance of genomic location and timing of Cdk2 expression and the possible compensation for loss of Cdk1 by Cdk2, we generated a mouse in which a *Cdk2* cDNA was knocked into the *Cdk1* locus (*Cdk1*^{Cdk2KI}). We found that substitution of both copies of *Cdk1* by *Cdk2* leads to early embryonic lethality, similar to deletion of *Cdk1*, even though the knockin Cdk2 is expressed from the *Cdk1* locus. In addition, in order to study the consequences of Cdk2 expression from the *Cdk1* locus on the function of Cdk2, we generated *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice, in which one copy of *Cdk2* and one copy of *Cdk1* are expressed from the *Cdk1* locus with a deletion of the *Cdk2* gene in the original *Cdk2* locus. From this study, we found that both male and female *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice are sterile, similar to *Cdk2*$^{-/-}$ mice, even though they express the Cdk2 protein from the *Cdk1* locus in testis.

MATERIALS AND METHODS

Generation of the Cdk1^{Cdk2KI} targeting vector

To construct the Cdk1^{Cdk2KI} targeting vector, we purchased a 129/Sv mouse genomic BAC clone harboring the genomic *Cdk1* locus (Resgene, pBeloBACII clone J21 plate 305; PKB576). Using BAC recombineering technology (Lee et al., 2001), we first inserted an FRT site into intron 2 of *Cdk1*. Then, a cassette harboring [*Cdk1* homology arm 5′-*Cdk2* cDNA-IRES-β-galactosidase-*FRT-loxP*-PGK-EM7-neomycin-poly(A)-*FRT-loxP Cdk1* homology arm 3′] was inserted in place of exon 3 of the *Cdk1* locus. The insertion site maintains all *Cdk1* exon 3 splicing sequences and results in a transcript including *Cdk1* exon 1 (5′ UTR), *Cdk1* exon 2 (including the ATG start codon plus 11 amino acids) and the *Cdk2*$^{\Delta 12}$*-HA* cDNA. We designed this construct to induce Cdk2^{12AACdk1} expression under potential regulatory sequences including the *Cdk1* 5′ UTR, promoter, intron 1 and intron 2. Moreover, over the first 12 amino acids, Cdk1 and Cdk2 are very similar [they differ by four amino acids (in red in Fig. 1A)], suggesting that this region would not affect Cdk2 properties (see Fig. S1 in the supplementary material). The *Cdk1*^{Cdk2KI} locus was then retrieved into pBluescriptLight-HSVTK (Liu et al., 2003) and, after recombination, a 22 kb fragment of the *Cdk1*^{Cdk2KI} locus was selected by ampicillin and kanamycin resistance. After electroporation and selection with G418 and gancyclovir, three independent embryonic stem cell clones were identified which had the *Cdk1*^{Cdk2KI} locus correctly targeted. Positive clones were screened by β-galactosidase expression, Southern blot and PCR. The following primers were used: 5′-ACCATGTATATGTTAGATCGTAG-3′ (PKO553), 5′-TCGCTTTCAAGTCTGATCTTCT-3′ (PKO554) and 5′-CGATATTAGGGTGATTAAGTTCC-3′ (PKO043). Wild-type clones yield a band of 300 bp, whereas the mutant clone produces a band of 450 bp. Germline transmission was obtained from two clones and these were used to generate chimeric animals.

Mice and surgical procedures

Mice were housed under standard conditions and were maintained on a 12-hour light/dark cycle. Mice were fed a standard chow diet containing 6% crude fat and were treated in compliance with the National Institutes of Health guidelines for animal care and use.

Twelve- to fifteen-week-old *Cdk2*$^{+/+}$, *Cdk2*$^{-/-}$, *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ male mice were used and all animals were operated upon under sterile conditions between 9 am and 12 pm, as described previously (Satyanarayana et al., 2003). Mice were anesthetized by intraperitoneal injection of avertin and were subjected to partial (70%) hepatectomy (PH). After 2 hours of BrdU labeling, mice were sacrificed at 24 (n=3), 48 (n=4) and 72 (n=4) hours after PH. For BrdU pulse labeling, 10 μl/g body weight of labeling reagent (10:1, 5-bromo-2-deoxyuridine:5-fluro-20-deoxyuridine; Cell Proliferation Kit RPN20, Amersham) was administered intraperitoneally 2 hours before sacrifice. After euthanizing the mice, portions of the liver lobes were fixed separately for BrdU, Hematoxylin and Eosin and β-galactosidase staining.

Preparation of mouse embryonic fibroblasts (MEFs) and cell culture

MEFs were prepared as described previously (Berthet et al., 2003) from E13.5 *Cdk2*$^{+/+}$, *Cdk2*$^{-/-}$, *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ embryos. Serum starvation and stimulation experiments were done as described previously (Satyanarayana et al., 2008).

Alamar Blue cell-proliferation assay

Proliferation of *Cdk2*$^{+/+}$, *Cdk2*$^{-/-}$, *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ MEFs in response to serum starvation (0.1% FBS) and stimulation (10% FBS) was analyzed in 96-well plates as described previously (Satyanarayana et al., 2008).

Immunocytochemistry and confocal microscopy

Serum-starved (DMEM medium with 0.1% FBS, 96 hours) and serum-stimulated (DMEM medium with 10% FBS) cells from 100-mm culture dishes were transferred on to coverslips in 12-well plates at a density of 1×10^5 cells per well and probed with specific antibodies at 0, 6, 12 and 24 hours after stimulation. Immunocytochemical staining was conducted as described (Satyanarayana et al., 2008). Primary antibodies against Cdk2 and HA-tag (to detect knockin Cdk2-HA) (Berthet et al., 2003) were used at 1:200 dilution. At each time point, the staining pattern was analyzed in several low-power fields (63×) and the images were captured with a confocal laser-scanning microscope (LSM510, Zeiss).

Immunohistochemistry

Slides were rehydrated and a microwave antigen-retrieval step was performed for 13 minutes in 10 mM sodium citrate (pH 6.0) containing 0.05% Tween 20. The sections were then treated with 3% hydrogen peroxide for 10 minutes. Blocking was carried out using 2.5% horse serum, 1% BSA in PBS for 30 minutes. Slides were incubated at room temperature for 1 hour with the following primary antibodies: Cdk2 (1:1000), Cdk1 (1:200), HA-Cdk2 (1:5000) (Berthet et al., 2003) and Cdk2 (Abcam). Antibody detection was achieved using the anti-rabbit ImmPRESS Reagent Kit (Vector Labs) according to the manufacturer's protocol. Slides were counterstained with Mayer's Hematoxylin, mounted with Permount mounting media, and coverslips were applied.

BrdU immunohistochemical staining

BrdU immunohistochemical staining on formalin (Sigma, HT50-1-128)-fixed 5-μm liver sections was performed as described (Satyanarayana et al., 2008). An Axioplan2 imaging microscope (Zeiss) was used to photograph and analyze the BrdU staining pattern (and likewise for H&E, β-galactosidase and apoptotic staining). At least 3000 nuclei were counted per slide and the percentage of BrdU-positive nuclei calculated.

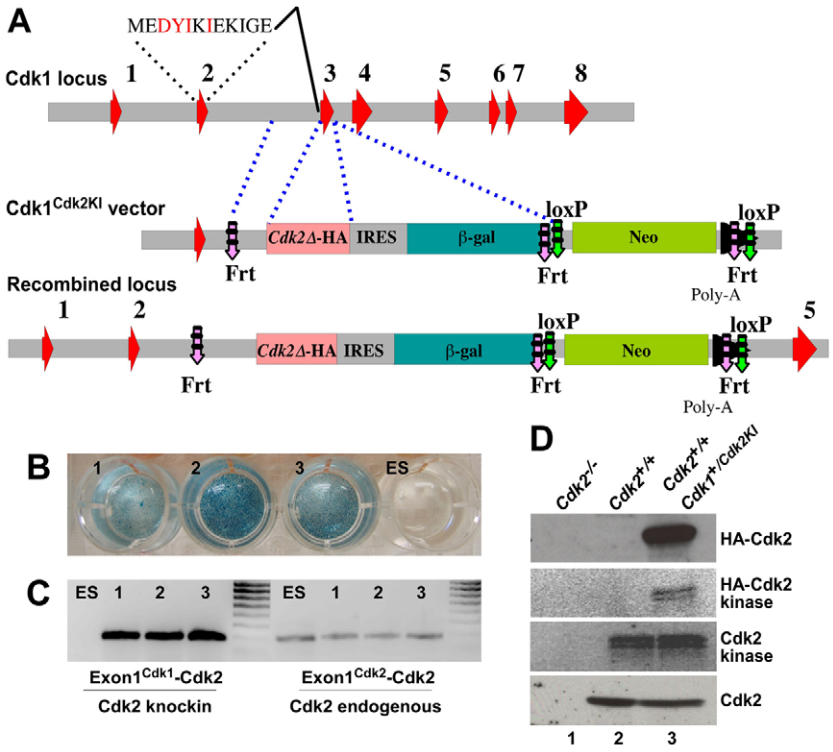

Fig. 1. The *Cdk1*^{Cdk2KI} knockin construct. (**A**) Schematic showing the strategy used to generate the *Cdk1*^{Cdk2KI} knockin construct (for details see Materials and methods). Exons (red arrowheads) are numbered. (**B**,**C**) Mouse embryonic stem (ES) cell clones positive for the *Cdk1*^{Cdk2KI} locus were screened for *lacZ* expression (β-galactosidase staining, blue) (B) and by PCR (C). Exon1^{Cdk2}-Cdk2 indicates endogenous *Cdk2*, whereas Exon1^{Cdk1}-Cdk2 indicates that exon 1 was from *Cdk1* and the remaining coding region from *Cdk2*. Three independent embryonic stem cell clones (1, 2, 3) are shown that had the *Cdk1*^{Cdk2KI} locus correctly targeted. (**D**) Western blots and histone H1 kinase assays indicating the expression level of knockin Cdk2-HA (top panel) and Cdk2 (bottom panel), and the kinase activity of knockin HA-tagged Cdk2 (second panel from top) and of endogenous Cdk2 (third panel from top) in *Cdk2*$^{-/-}$ (lane 1), *Cdk2*$^{+/+}$ (lane 2) and *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (lane 3) MEFs.

Hematoxylin and Eosin (H&E) staining

Frozen sections of liver, testes, ovaries and embryos were warmed to room temperature for ~20 minutes. The tissue sections were fixed in acetone for 10 minutes and then air dried. Slides were rinsed with distilled water (2 minutes), incubated in Hematoxylin (Richard-Allan Scientific, 7231) for 3 minutes, and then washed with distilled water twice for 2 minutes. The slides were treated with clarifier (Richard-Allan Scientific, 7402) for 2 minutes, followed by a brief wash with distilled water. After immersing the slides in Bluing Reagent (Richard-Allan Scientific, 7301) for 1 minute, they were washed with water (2 minutes), incubated in 95% ethanol for 1 minute, and then with Eosin Y (Richard-Allan Scientific, 7111) for 20 seconds. Then, the slides were incubated in 100% ethanol (three times, 1 minute each), followed by xylene (three times, 1 minute each).

β-galactosidase staining

The tissues or embryos were fixed in formaldehyde/glutaraldehyde fixative [192.6 ml PBS, 5.4 ml 37% formaldehyde, 1.6 ml 25% glutaraldehyde, 0.4 ml IGEPAL (NP40 substitute; Sigma, I3021)]. Sections (10 μm) were prepared from these fixed samples. The slides were rinsed three times for 5 minutes each in PBS. After placing the slides in a humid chamber, the β-galactosidase staining solution [17.1 ml PBS, 0.5 ml X-gal (40 mg/ml in DMSO), 0.5 ml 100 mM $K_3Fe(CN)_6$ (Fluka, 60299), 0.5 ml 100 mM $K_4Fe(CN)_6$ (Fluka, 60279), 40 μl 1 M $MgCl_2$] was applied directly onto the tissue sections and incubated overnight at 31°C in the dark. After washing, the slides were counterstained in a 0.1% Neutral Red solution for 30 seconds. The sections were dehydrated in 100% ethanol and then incubated three times in xylene, 1 minute each.

Apoptotic staining

Testes were fixed in 10% formalin (NBF; Sigma, HT50-1-128). Apoptotic staining followed the manufacturer's protocol (Chemicon, S7100).

Immunoblotting and kinase assays

Whole-cell lysates from passage-three MEFs were prepared as described (Berthet et al., 2003). For western blotting, 50 μg of protein was separated on 12.5% polyacrylamide gels (Bio-Rad), transferred onto Immobilon-P transfer membranes (Millipore, IPVH00010) using semi-dry blotting, and probed with the following primary antibodies: Cdk2, Cdk1, Cdk4, HA-Cdk2, cyclin B1 as described previously (Berthet et al., 2003), cyclin E1 (gift of Bruno Amati, European Institute of Oncology, Milan, Italy), cyclin D1 (Neomarkers, RB-010p), p27 (Zymed, 71-9600) and actin (Santa Cruz, C0306). All antibodies were used at 1:1000. For kinase assays (Cdk2 and HA-Cdk2), 250 μg of protein from cell lysates and 7 μl of anti-Cdk2 antibody-coupled agarose A beads [as described by Berthet et al. (Berthet et al., 2003)] or HA-antibody-coupled agarose A beads (Roche, 11815016001) were used and the kinase assays performed as described previously (Aleem et al., 2005). For co-immunoprecipitation assays (HA-Cdk2/cyclin E1, HA-Cdk2/cyclin A2), 400 μg of protein from cell lysates and 7 μl of HA-coupled agarose A beads were used.

RESULTS

Cdk1 substitution by Cdk2 is lethal

Recently, we reported that Cdk1 can bind to cyclin E and take over the functions of Cdk2 in its absence to drive cells through the G1–S transition (Aleem et al., 2005). To explore the reverse situation, i.e. whether Cdk2 can functionally substitute for Cdk1, we knocked the *Cdk2* cDNA into the *Cdk1* locus (Fig. 1A). The knockin construct was electroporated into embryonic stem (ES) cells. The homologous recombination event in heterozygous ES cells was identified by *lacZ* reporter gene expression (Fig. 1B), PCR (Fig. 1C) and Southern blot (data not shown). Heterozygous ES cells were injected into mouse blastocysts to generate chimeras. The chimeric mice were backcrossed to produce *Cdk1*$^{+/Cdk2KI}$ heterozygous mice. At this point, mouse embryonic fibroblasts (MEFs) were prepared from E13.5 embryos of *Cdk1*$^{+/Cdk2KI}$ mice and the expression and kinase activity of knockin HA-tagged Cdk2 was analyzed. This analysis indicated that knockin HA-Cdk2 was expressed and displayed kinase activity similar to that of endogenous Cdk2 (Fig. 1D). Heterozygous mice were then intercrossed to generate homozygous *Cdk1*$^{Cdk2KI/Cdk2KI}$ mice. Out of 258 mice analyzed, 88 (34%) were *Cdk1*$^{+/+}$ and 170 (66%) were *Cdk1*$^{+/Cdk2KI}$, but no *Cdk1*$^{Cdk2KI/Cdk2KI}$ mice were obtained. In addition, a total of 143 embryos were analyzed at E9.5 (n=23), E10.5 (n=19), E12.5 (n=23), E13.5 (n=35), E14.5 (n=16), E16.5 (n=13) and E18.5 (n=14). All embryos analyzed were either *Cdk1*$^{+/+}$ or *Cdk1*$^{+/Cdk2KI}$ and

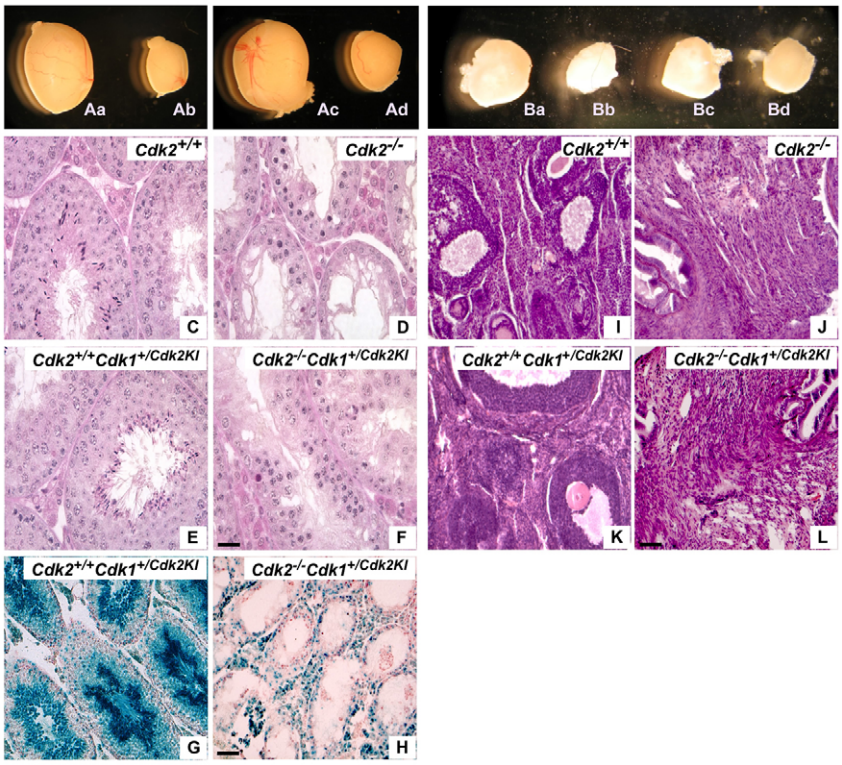

Fig. 2. Genetically relocated Cdk2 loses its meiotic function. (**A**) Testes from P90 *Cdk2*$^{+/+}$ (Aa), *Cdk2*$^{-/-}$ (Ab), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Ac) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Ad) mice. (**B**) Ovaries from P90 *Cdk2*$^{+/+}$ (Ba), *Cdk2*$^{-/-}$ (Bb), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Bc) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Bd) mice. (**C-F**) H&E-stained testes sections from P90 *Cdk2*$^{+/+}$ (C), *Cdk2*$^{-/-}$ (D), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (E) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (F) mice. (**G**,**H**) Testes sections, stained for β-galactosidase, from *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (G) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (H) mice. (**I-L**) H&E-stained ovary sections from P90 *Cdk2*$^{+/+}$ (I), *Cdk2*$^{-/-}$ (J), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (K) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (L) mice. Representative pictures are presented. Scale bars: 20 μm in C-F; 50 μm in G-L.

none displayed the *Cdk1*$^{Cdk2KI/Cdk2KI}$ genotype. Also, 69 blastocyts were recovered from the uteri of four *Cdk1*$^{+/Cdk2KI}$ super-ovulated females and from six *Cdk1*$^{+/Cdk2KI}$ naturally bred females with *Cdk1*$^{+/Cdk2KI}$ male mice. None of these blastocysts displayed any abnormal phenotypes, such as fragmentation, shrinkage or degeneration, but all blastocysts genotyped were either *Cdk1*$^{+/+}$ or *Cdk1*$^{+/Cdk2KI}$ and none was homozygous for *Cdk1*^{Cdk2KI}. However, when we crossed *Cdk1*$^{+/Cdk2KI}$ male or female mice with C57BL6 wild-type mice, both wild-type and *Cdk1*$^{+/Cdk2KI}$ litters were obtained at the expected frequency. This indicates that the *Cdk1*^{Cdk2KI} germ cells are viable and functional, but that the fertilized homozygous embryos are unable to reach the blastocyst stage. Our analysis suggests that the substitution of Cdk1 by Cdk2 leads to early embryonic lethality before E3.5, comparable to the effect of deleting the *Cdk1* gene or a gene-trap mutation in the *Cdk1* locus (our unpublished results) (Santamaria et al., 2007). To test whether the loss of p53 (Trp53 – Mouse Genome Informatics) could rescue the phenotype of *Cdk1*$^{Cdk2KI/Cdk2KI}$ mice, *Cdk1*$^{+/Cdk2KI}$ mice were crossed with *p53*$^{-/-}$ mice. We did not obtain any mice, embryos or blastocysts with the *p53*$^{-/-}$ *Cdk1*$^{Cdk2KI/Cdk2KI}$ genotype (data not shown). This indicates that loss of p53 does not rescue the phenotype of *Cdk1*$^{Cdk2KI/Cdk2KI}$ mice and that at least one copy of *Cdk1* is essential for survival.

Cdk2 loses its meiotic function when expressed from the *Cdk1* locus

To identify the consequences for the function of Cdk2 when expressed from the *Cdk1* locus, we crossed *Cdk1*$^{+/Cdk2KI}$ mice with *Cdk2*$^{+/-}$ mice. From such crosses we obtained *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice, in which two copies of *Cdk2* are expressed from the original *Cdk2* locus and one copy of knockin *Cdk2* from the *Cdk1* locus, as well as *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice, in which only one copy of knockin *Cdk2* is expressed from the *Cdk1* locus and *Cdk2* expression from the endogenous locus is abolished. Littermate *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice did not display any morphological differences. Similarly, no significant differences were observed between *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice in the histopathology of any of the tissues analyzed (data not shown). Surprisingly, male and female *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice were sterile, similar to *Cdk2*$^{-/-}$ mice (Berthet et al., 2003; Ortega et al., 2003), even though they express a functional copy of *Cdk2* from the *Cdk1* locus. The testes and ovaries of adult *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice were atrophic and were only about half the size of those of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice (Fig. 2A,B). To determine the cause of the sterility, a histological analysis was conducted on post-natal day 90 (P90) ovaries of *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice. In *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ ovaries, the development of oocytes was normal and the correct follicle stages were observed (Fig. 2I,K). By contrast, in *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ ovaries, no follicles were observed and the oocytes failed to develop in the atrophic ovaries, similar to what is observed in *Cdk2*$^{-/-}$ females (Fig. 2J,L) (Berthet et al., 2003; Ortega et al., 2003). A histological analysis was also conducted on sexually immature and mature testes of *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice. This revealed that in the testes of adult *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice (P90), the size of the seminiferous tubules was much smaller than normal, resulting from a substantial depletion of spermatocytes (Fig. 2C-F), similar to that reported previously for *Cdk2*$^{-/-}$ mice (Berthet et al., 2003; Ortega et al., 2003). The numbers of spermatogonia and Sertoli cells were not affected in testes of *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice (Fig. 2F). In addition, we took advantage of the *lacZ* reporter in the *Cdk2* knockin construct and analyzed the expression of knockin Cdk2 from the *Cdk1* locus by β-galactosidase staining in testes of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice. We found that *lacZ* was abundantly expressed in the testes of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice and extensive β-galactosidase staining was observed in the spermatocytes (Fig. 2G). By contrast, in *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice, no expression was detected in the seminiferous tubules owing to the substantial depletion of spermatocytes (Fig. 2H), but faint β-galactosidase staining was observed in the testes (Fig. 2H).

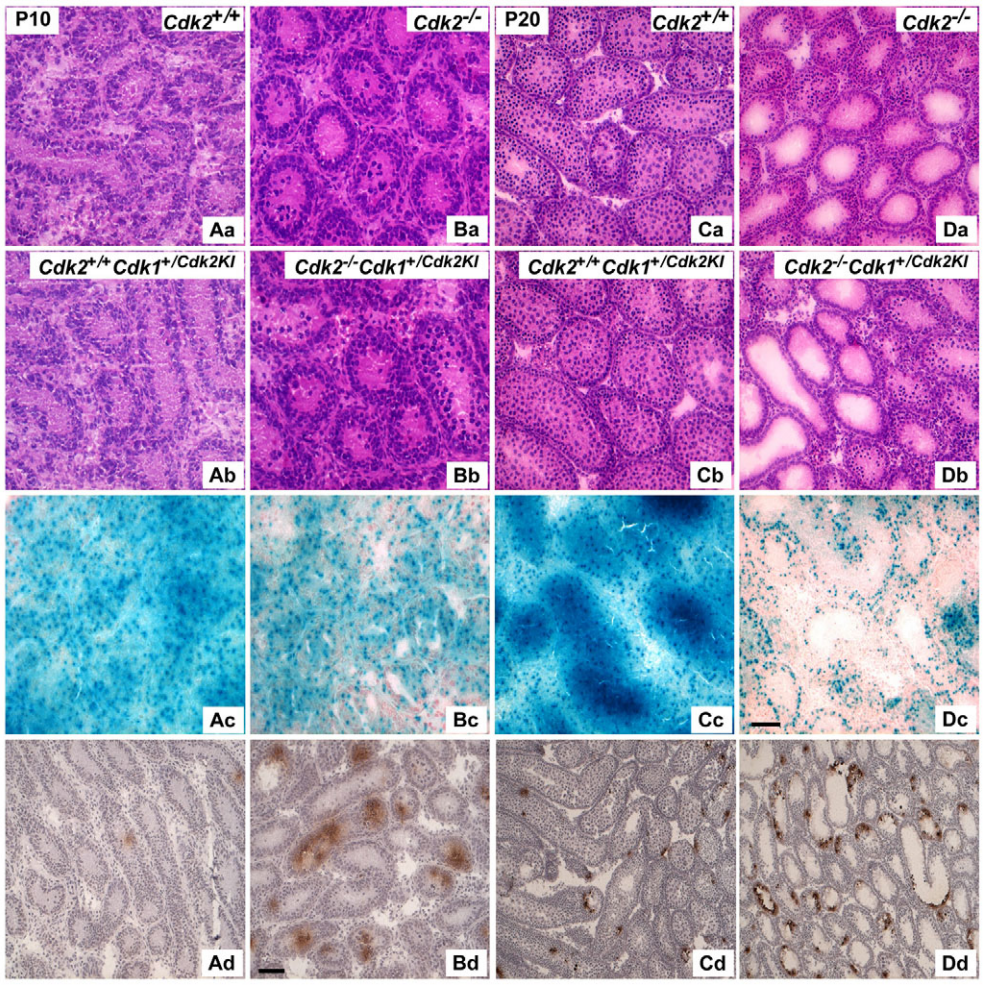

Fig. 3. Knockin Cdk2 fails to function in meiosis. (**A**,**B**) H&E-stained testes sections from P10 *Cdk2*$^{+/+}$ (Aa), *Cdk2*$^{-/-}$ (Ba), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Ab) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Bb) mice. Testes sections, stained for β-galactosidase, from P10 *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Ac) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Bc) mice. Apoptotic testes sections from P10 *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Ad) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Bd) mice. (**C**,**D**) H&E-stained testes sections from P20 *Cdk2*$^{+/+}$ (Ca), *Cdk2*$^{-/-}$ (Da), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Cb) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Db) mice. Testes sections, stained for β-galactosidase, from P20 *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Cc) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Dc) mice. Apoptotic testes sections from P20 *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Cd) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Dd) mice. Scale bars: 40 μm, except 100 μm in Ad,Bd,Cd,Dd.

In addition to adult testes, we also performed a histological analysis of testes from P10 and P20 *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice. Hematoxylin and Eosin staining of P10 testes revealed no significant differences between *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice, which were similar to *Cdk2*$^{-/-}$ and *Cdk2*$^{+/+}$ mice, respectively, as reported previously (Fig. 3Aa,b,Ba,b) (Ortega et al., 2003). We observed a similar expression pattern of knockin Cdk2 (β-galactosidase staining) in the P10 testes of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice (Fig. 3Ac,Bc). Nevertheless, we detected a marked increase in apoptosis of primary spermatocytes in P10 testes of *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ as compared with *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice (Fig. 3Ad,Bd). In contrast to P10 testes, visible defects were observed in P20 testes of *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ as compared with *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice (Fig. 3Ca,b,Da,b). At this stage in development, the first wave of germ cells is completing the second meiotic division and developing into round spermatids. Earlier stages of spermatogenesis can also be detected in tubules of P20 mice. P20 *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ testes were ~20-30% smaller than *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ testes (data not shown). Histological analysis revealed the absence of round spermatids in P20 *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ testes, similar to *Cdk2*$^{-/-}$ testes (Fig. 3Da,b). In addition, we found extensive germ cell apoptosis in P20 *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ as compared with *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ testes (Fig. 3Cd,Dd). In accordance with this germ cell apoptosis and depletion of spermatocytes, diminished expression of knockin Cdk2 (β-galactosidase) was detected in P20 *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Fig. 3Dc) as compared with *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Fig. 3Cc) testes.

Cdk2 expressed from the *Cdk1* locus partially rescues the *Cdk2*$^{-/-}$ meiotic phenotype

To determine why knockin Cdk2 fails to participate in and complete meiosis, we analyzed its expression and localization further in adult testes. A thorough analysis of 3-month-old adult testes revealed that *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ spermatocytes were able to reach further stages of meiosis than *Cdk2*$^{-/-}$ spermatocytes. In *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ testes, there was a greater number of spermatocytes reaching the pachytene stage of meiosis (arrows in Fig. 4Da, Fig. 2F) than in *Cdk2*$^{-/-}$ testes, where cells with pachytene morphology and condensed sex body were rarely observed (arrows in Fig. 4Ca, Fig. 2D). A possible explanation for the lack of full rescue is that Cdk2 was not being expressed at the appropriate time from the *Cdk1* locus. In order to explore this possibility, we analyzed the expression of Cdk1, Cdk2 and knockin Cdk2 in wild-type and mutant testes. The localization of Cdk1 in wild-type testes was cytoplasmic in spermatogonia but was decreased and possibly absent in leptotene and zygotene cells (arrows, Fig. 4Aa,Ba). A resumption of Cdk1 expression was evident in the nuclei and cytoplasm of pachytene spermatocytes, but Cdk1 was not detectable in round spermatids. In *Cdk2*$^{-/-}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ testes, Cdk1 was expressed in nearly all cells present except Sertoli cells (Fig. 4Ca,Da). Wild-type testes showed weak cytoplasmic staining of Cdk2 in spermatogonia and faint-to-no staining in leptotene and zygotene cells (arrows, Fig. 4Ab). By contrast, pachytene spermatocytes displayed solid Cdk2 staining in the nucleus. Additionally, some round spermatids were strongly positive for Cdk2. In contrast to *Cdk2*$^{+/+}$ testes, robust

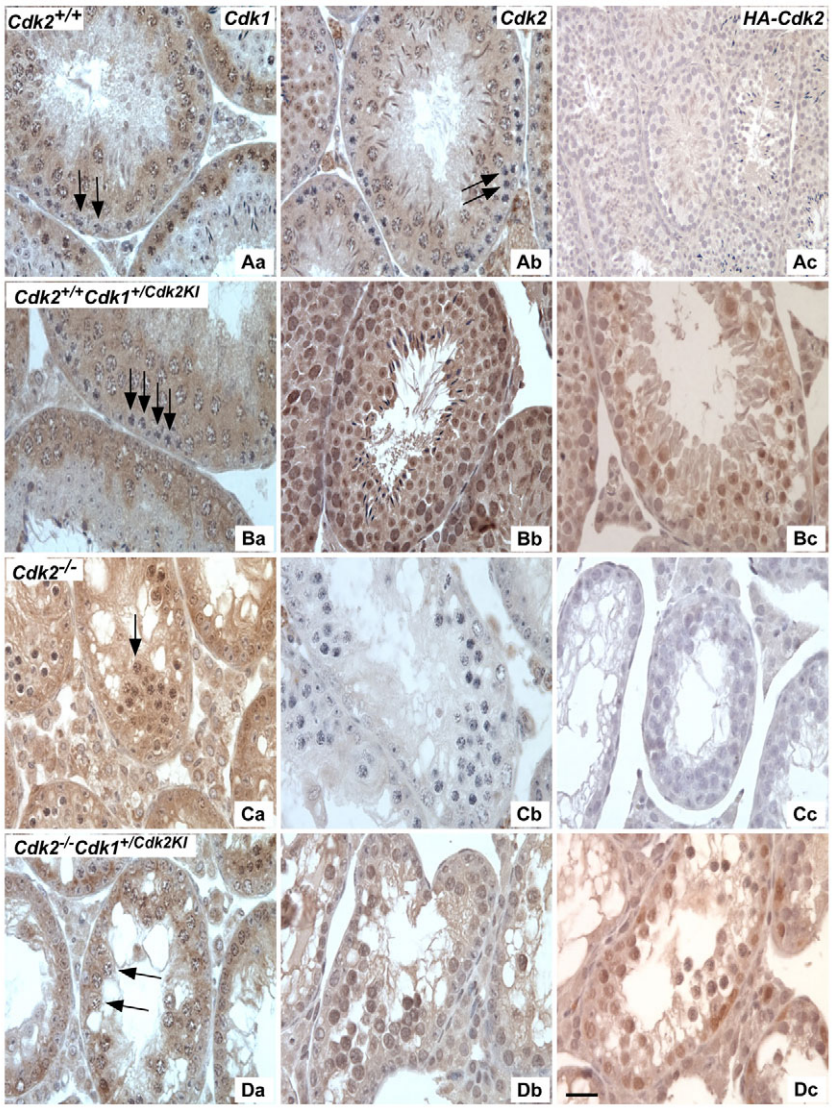

Fig. 4. Knockin Cdk2 expression is insufficient to rescue the *Cdk2*$^{-/-}$ meiotic defect. (**A-D**) Immunohistochemistry of Cdk1, Cdk2 and HA (Cdk2KI) on testes from 3-month-old mice. Sections stained for Cdk1: *Cdk2*$^{+/+}$ (Aa), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Ba), *Cdk2*$^{-/-}$ (Ca) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Da). Sections stained for Cdk2: *Cdk2*$^{+/+}$ (Ab), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Bb), *Cdk2*$^{-/-}$ (Cb) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Db). Sections stained for HA: *Cdk2*$^{+/+}$ (Ac), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Bc), *Cdk2*$^{-/-}$ (Cc) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Dc). Arrows in Aa and Ba indicate leptotene and zygotene spermatocytes. Arrows in Ca and Da indicate cells with a pachytene-like morphology. Arrows in Ab indicate cells with zygotene morphology. Scale bar: 20 μm.

staining of Cdk2 was detected in all cell types in *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ testes. This could be due to the fact that the endogenous and knockin Cdk2 were expressed at different stages of meiosis and at least one of the two forms was present in the cells (Fig. 4Bb). When we used HA antibodies to detect knockin Cdk2, the expression was limited to only a few cells (Fig. 4Bc). We did not detect any Cdk2 staining in *Cdk2*$^{-/-}$ testes, as expected (Fig. 4Cb). Surprisingly, the knockin Cdk2 was present in nearly all remaining cells in testes of *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice (Fig. 4Dc). This expression was confirmed by HA antibody staining (Fig. 4Bc,Dc); neither wild-type nor *Cdk2*$^{-/-}$ mice stained for HA (Fig. 4Ac,Cc).

Expression of Cdk2 from the *Cdk1* locus does not affect the localization or cell cycle properties of Cdk2

Previous studies in wild-type mouse cells reported that Cdk2 is predominantly present in the nucleus, irrespective of the cell cycle stage (Moore et al., 1999; Satyanarayana et al., 2008). By contrast, Cdk1 is present primarily in the cytoplasm and translocates to the nucleus only during mitosis after nuclear breakdown (Bailly et al., 1989; Bailly et al., 1992). To identify the consequences of genetic replacement for the translocational properties of Cdk2 we monitored, by immunofluorescence, the localization of endogenous Cdk2 and knockin Cdk2 (expressed from the *Cdk1* locus) in serum-starved and serum-stimulated *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ MEFs at different stages of the cell cycle (Fig. 5Aa-Bh). Our analysis revealed that endogenous Cdk2, as well as HA-tagged knockin Cdk2 expressed from the *Cdk1* locus, were predominantly present in the nucleus in serum-starved cells, although we also found a certain amount of staining in the cytoplasm for both endogenous and knockin Cdk2 (Fig. 5Ae,Be). Between 6 and 24 hours after serum stimulation, we detected the endogenous or knockin Cdk2 primarily localized in the nucleus (Fig. 5Af-Ah,Bf-Bh). Similarly, when we monitored the localization of knockin Cdk2 in the absence of endogenous Cdk2 in *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ MEFs, we did not observe any difference in the localization pattern of knockin Cdk2, as it was present predominantly in the nucleus even though it was expressed from the *Cdk1* locus (data not shown).

To identify whether cells expressing three copies of *Cdk2* had any proliferative advantage over wild-type or *Cdk2*$^{-/-}$ MEFs, we measured the proliferation rate of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$, *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs. Our analysis indicated that there was no significant difference in the proliferation rate of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ as compared with *Cdk2*$^{+/+}$ MEFs, even though they express an extra

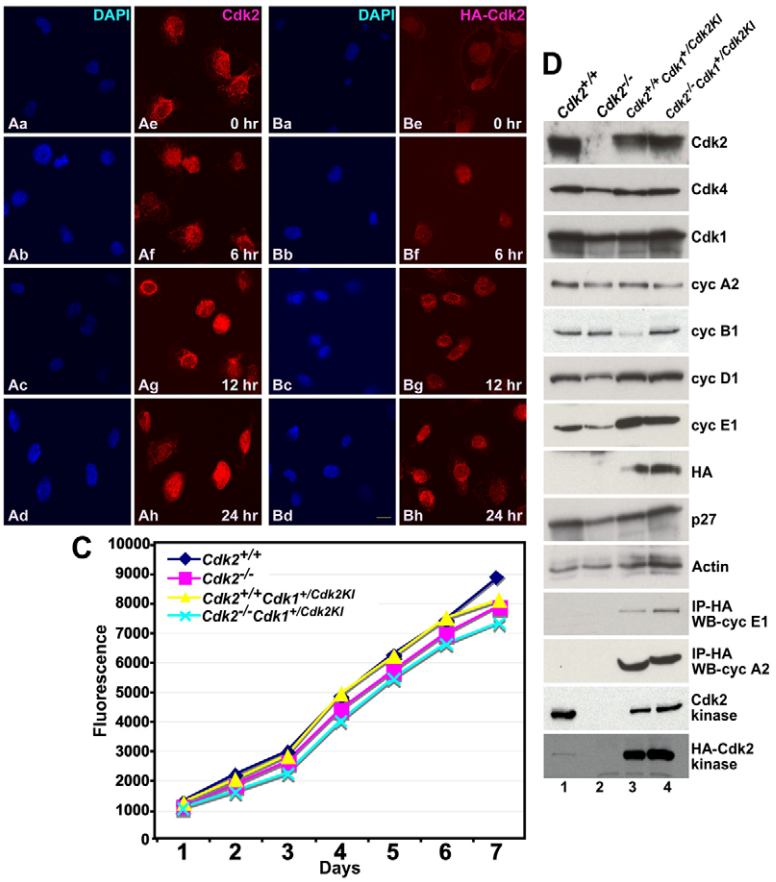

Fig. 5. Genetically replaced Cdk2 retains its normal subcellular localization. (**A**,**B**) Immunofluorescence staining of Cdk2 and HA-Cdk2 in serum-starved and serum-stimulated *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ MEFs at different time points. Cdk2 and HA-Cdk2 localization was detected by rabbit anti-Cdk2 and rabbit anti-HA followed by Alexa Fluor 568-conjugated goat anti-rabbit antibodies (red; Ae-h, Be-h), with the nuclei counterstained with DAPI (blue; Aa-d, Ba-d). The images were captured with a laser confocal microscope (63×). These representative pictures are from one of the three independent stainings. Scale bar: 10 μm. (**C**) Line graph displaying the proliferation rate of *Cdk2*$^{+/+}$, *Cdk2*$^{-/-}$, *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ MEFs. (**D**) Western blots showing the expression of Cdk2, Cdk4, Cdk1, cyclin A2, cyclin B1, cyclin D1, cyclin E1, HA-Cdk2, p27 (panels 1-9 from top, respectively), HA-Cdk2/cyclin E1 and HA-Cdk1/cyclin A2 co-immunoprecipitations (panels 11 and 12 from top, respectively), as well as the histone H1 kinase activity of Cdk2 and HA-Cdk2 (Cdk2KI) (bottom two panels) in *Cdk2*$^{+/+}$ (lane 1) *Cdk2*$^{-/-}$ (lane 2), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (lane 3) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (lane 4) MEFs. Actin (panel 10) served as a loading control.

copy of *Cdk2* from the *Cdk1* locus (Fig. 5C). Similarly, we did not observe any significant difference in the proliferation rate of *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ MEFs as compared with *Cdk2*$^{+/+}$ MEFs or those of the other two genotypes (Fig. 5C). Co-immunoprecipitation assays revealed that the knockin HA-tagged Cdk2 was able to form a complex with cyclin E1 (Fig. 5D, eleventh panel from the top) and cyclin A2 (Fig. 5D, twelfth panel), similar to endogenous Cdk2 as described previously (Elledge et al., 1992; Sheaff et al., 1997). In addition, when we determined the expression pattern of some of the Cdks and cyclins that play a role in the G1, S and G2 phases of the cell cycle, we did not observe any significant differences in their expression levels between *Cdk2*$^{+/+}$, *Cdk2*$^{-/-}$, *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ genotypes (Fig. 5D), with the exception of an increase in cyclin E expression when Cdk2KI was present (Fig. 5D, lanes 3 and 4). This indicates that the expression of Cdk2 from the *Cdk1* locus did not affect its cell cycle functions, and that the presence of an extra copy of *Cdk2*, or the loss of one copy of *Cdk1*, does not have any impact on the cell cycle.

Loss of Cdk2 leads to premature transcriptional activation of *Cdk1*

To identify the consequences of loss of Cdk2 on the transcriptional activation of *Cdk1*, we used *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice, taking advantage of the *lacZ* reporter gene in our target vector to follow the transcriptional activation of *Cdk1* in vivo by β-galactosidase staining. We employed the well-established liver regeneration after partial hepatectomy (PH) model system (Fausto, 2000; Kountouras et al., 2001) to study the transcriptional activation of *Cdk1* during in vivo cell cycle initiation and progression in the presence or absence of Cdk2 (Fig. 6Aa-Bd). In response to PH, S-phase initiation occurs at 24 hours, peaks at 48 hours, and the first round of replication is completed at ~72 hours (Fausto, 2000; Satyanarayana et al., 2004). In the presence of Cdk2 (*Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$), initiation of *Cdk1* transcription occurred 24 hours after PH, as revealed by weak β-galactosidase staining in the regenerating liver (Fig. 6Ab). At 48 hours after PH, β-galactosidase staining was stronger than at 24 hours and ~50% of the cells stained for β-galactosidase (Fig. 6Ac). At 72 hours after PH, more than 90% of the cells displayed β-galactosidase staining and the staining pattern was even stronger than at earlier time points (Fig. 6Ad). This analysis suggests that the increase in the staining pattern was due not only to increased transcriptional activation, but also to the accumulation of more protein. In contrast to the transcriptional activation of *Cdk1* in *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice, *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice displayed premature transcriptional activation of *Cdk1* as revealed by a robust β-galactosidase staining pattern at 24 hours after PH, when ~40% of the cells already stained for β-galactosidase (Fig. 6Bb). At later time points (48 hours after PH), the staining pattern appeared more intense (~70% of cells β-galactosidase-positive) than in *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice (Fig. 6Bc). However, at 72 hours, the β-galactosidase staining pattern was similar in *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice (Fig. 6Bd).

In addition to monitoring the transcriptional activation of *Cdk1* by β-galactosidase staining, we also monitored the initiation and progression of the cell cycle 24 to 72 hours after PH in *Cdk2*$^{+/+}$, *Cdk2*$^{-/-}$, *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice. We observed that S-phase was slightly delayed in *Cdk2*$^{-/-}$ as compared with *Cdk2*$^{+/+}$ mice, especially at 24 hours (Fig. 6Cb,Db,E), as reported previously (Satyanarayana et al., 2008). The initiation and peak of S-phase were not altered, but the percentage of BrdU-

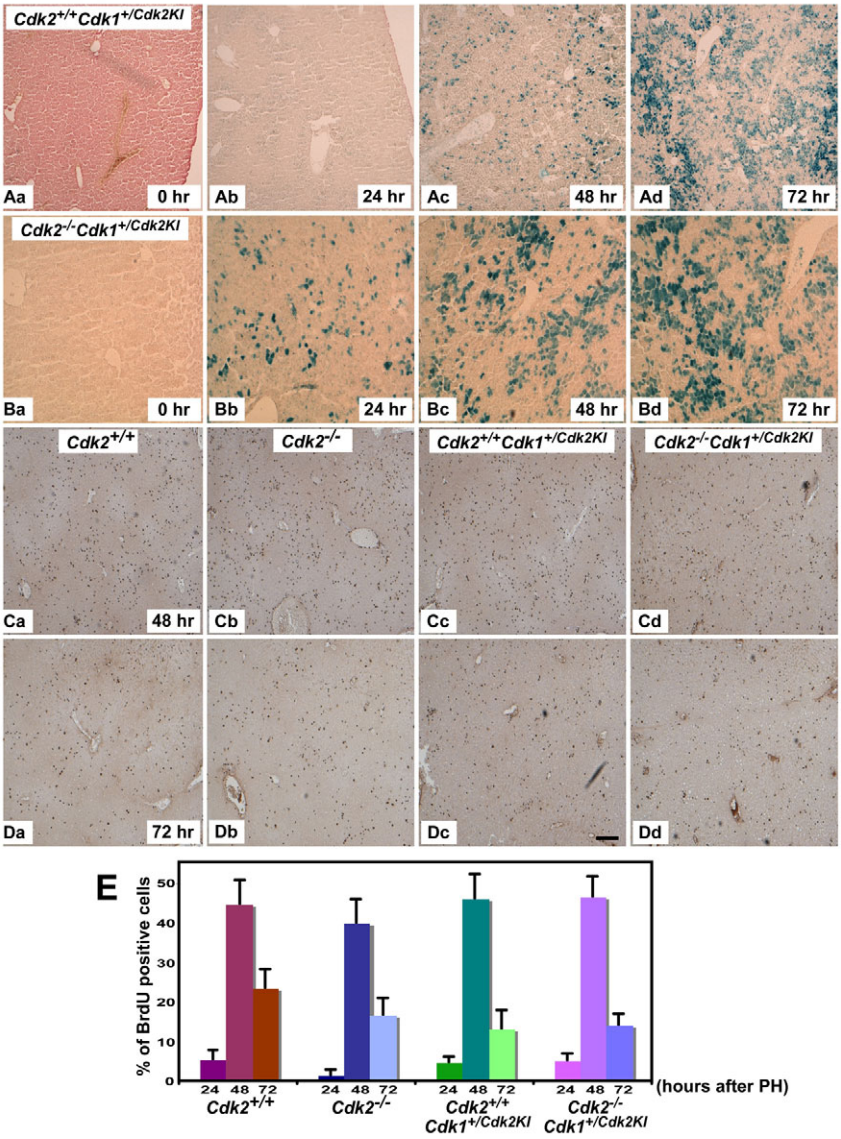

Fig. 6. Loss of Cdk2 leads to premature transcriptional activation of *Cdk1*. (**A**,**B**) The β-galactosidase staining pattern in regenerating livers of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Aa-d) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Ba-d) mice at the indicated time points. (**C**,**D**) Immunohistochemical BrdU staining pattern in the regenerating livers of *Cdk2*$^{+/+}$ (Ca,Da), *Cdk2*$^{-/-}$ (Cb,Db), *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (Cc,Dc) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (Cd,Dd) mice at the indicated time points. (**E**) Bar chart showing the percentage of BrdU-positive liver cells at the indicated time points in regenerating livers of *Cdk2*$^{+/+}$, *Cdk2*$^{-/-}$, *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice after 70% PH. The average and s.d. (error bars) were calcualted after counting a minimum of 3000 nuclei from several low-power fields per sample from each genotype. For each time point, four mice were analysed. Scale bar: 100 μm.

positive cells was decreased at 24 (and 48) hours after PH in *Cdk2*$^{-/-}$ regenerating livers as compared with *Cdk2*$^{+/+}$ livers (Fig. 6Cb,E). In contrast to *Cdk2*$^{-/-}$ mice, *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice did not display any difference in the regenerative response as compared with *Cdk2*$^{+/+}$ mice, and the percentage of BrdU-positive cells between 24 and 72 hours after PH was similar to that of *Cdk2*$^{+/+}$ or *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice (Fig. 6Ca,c,d,Da,c,d,E). This indicates that the knockin Cdk2 expressed from the *Cdk1* locus is able to mimic the cell cycle function of endogenous Cdk2. In addition, it appears that the presence of an extra copy of *Cdk2* in *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice did not confer any proliferative advantage, and one copy of *Cdk1* was sufficient for normal liver regeneration after PH. The differential transcriptional activation of *Cdk1* during different stages of liver regeneration prompted us to explore the transcriptional activation of *Cdk1* during embryogenesis and in adult tissues.

Variable transcriptional activation of *Cdk1* during embryogenesis and in adult tissues

The presence of the *lacZ* reporter gene in the targeting vector allowed us to explore the transcriptional activation of *Cdk1* during different stages of embryogenesis. Similarly, we monitored the activity status of *Cdk1* in most of the adult tissues of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice. *Cdk1* transcriptional activation was observed in most of the organs of the embryo between E14.5 and E20.5 (Fig. 7C′-F″). During earlier stages of embryogenesis (E12.5 and E13.5), a faint β-galactosidase staining was detected predominantly in the abdominal region, including the liver, kidney, different components of the digestive system, in the heart and lungs (Fig. 7A′-B″). By contrast, no β-galactosidase staining was detected in E9.5 embryos (data not shown). As a result of the ubiquitous expression of *Cdk1* between E14.5 and E20.5, no significant difference in the transcriptional activation of *Cdk1* between *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ (in the presence of Cdk2) and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ (in the absence of Cdk2) embryos was detected (data not shown). Owing to the design of the knockin construct with an IRES/β-gal cassette, it is possible that the transcriptional activity of the *Cdk1* locus was underestimated by a factor of up to 10, which would explain the low staining in early embryos.

Furthermore, when we analyzed the transcriptional activation of *Cdk1* by *lacZ* expression in several adult tissues in *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice, we did not observe expression of Cdk1 (β-galactosidase staining) in most of the tissues, including brain, heart,

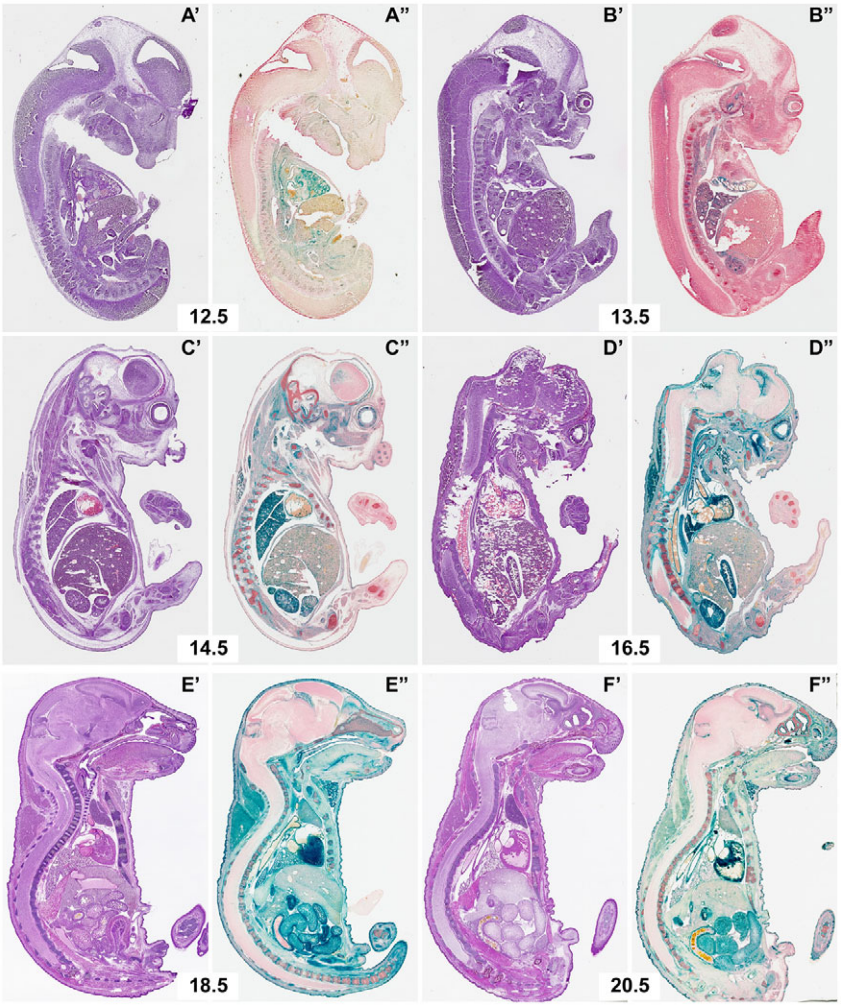

Fig. 7. Transcriptional activation of *Cdk1* during embryogenesis. (**A-F**) H&E (A′,B′,C′,D′,E′,F′) and β-galactosidase (A″,B″,C″,D″,E″,F″) staining patterns during different stages of embryogenesis in *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice. At each stage, four to five embryos were sectioned.

liver, lung, kidney and skin (Fig. 8A-E). In the case of the thymus, β-galactosidase staining was mainly observed in the medulla (Fig. 8F′,F″). In spleen, β-galactosidase staining was detected in the hematogenous red pulp (Fig. 8G,G″). In contrast to these other organs, robust expression of Cdk1 was observed in testis: spermatids, spermatocytes and Sertoli cells were solidly stained for β-galactosidase (Fig. 8H′,H″). This observation is in accordance with previous reports that Cdk1 is widely expressed in germ cells (see Ravnik and Wolgemuth, 1999) (see Fig. 4Aa,Ba,Ca,Da). When we analyzed the expression level of endogenous Cdk2 and HA-tagged knockin Cdk2 in different tissues of adult *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice, expression was absent in most of the adult tissues, except for spleen, testes and thymus (Fig. 8I).

DISCUSSION

It has been hypothesized that the presence of multiple Cdks in mammalian cells poses additional levels of control during cell cycle initiation and progression and that certain Cdk/cyclin combinations perform tissue-specific functions (Aleem and Kaldis, 2006; Pagano and Jackson, 2004). Furthermore, it was suggested that the availability of multiple Cdks in mammalian cells offers compensatory mechanisms in the absence of one or more Cdks. In support of this hypothesis, a single deletion of *Cdk2*, *Cdk4* or *Cdk6* does not affect the survival of mice (Berthet et al., 2003; Malumbres et al., 2004; Ortega et al., 2003). In addition, it was shown that double knockout of *Cdk4* and *Cdk6*, or of *Cdk2* and *Cdk4*, leads to embryonic lethality (Berthet et al., 2006; Malumbres et al., 2004; Santamaria et al., 2007). These studies imply that the loss of one or two Cdks is compensated partially or completely by other Cdk-cyclin complexes. Notably, Cdk1 compensates for the loss of Cdk2 by complexing with cyclin E (Aleem et al., 2005). Nevertheless, compensation of Cdk2 by Cdk1 appears to be only partial as *Cdk2* knockout males and females are sterile (Berthet et al., 2003; Ortega et al., 2003). This indicates that Cdk1 cannot fulfil the meiotic functions of Cdk2. To date, whether any of the Cdks can substitute for the functions of Cdk1 has not been explored. The fact that Cdk1, although a mitotic kinase, is able to perform the functions of the S-phase kinase Cdk2, raises the possibility that Cdk2 might substitute for the loss of Cdk1. This hypothesis is strengthened further by the observation that Cdk2 can bind to cyclin B1 (Aleem et al., 2005).

Contrary to this hypothesis, it has been reported that the deletion of *Cdk1* leads to early embryonic lethality, with embryos dying before E3.5 (Santamaria et al., 2007) (our unpublished results). This indicates that none of the Cdks can compensate for the loss of Cdk1 in terms of lethality. We hypothesized that if the timing of transcriptional activation and the genomic location of *Cdk2* match those of *Cdk1*, Cdk2 might acquire some of the properties of Cdk1 and thereby compensate for the loss of Cdk1. However, even when Cdk2 was expressed from the *Cdk1* locus, we did not observe any rescue of the lethality. We found that

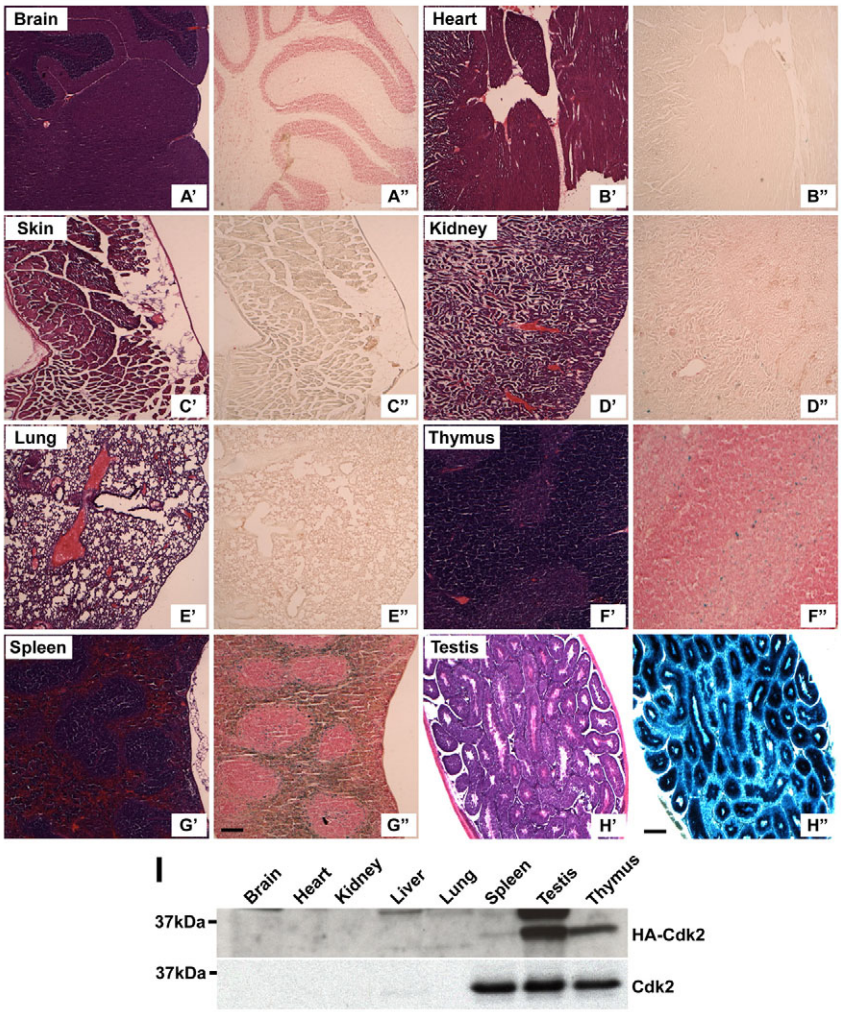

Fig. 8. Transcriptional activation of *Cdk1* in adult tissues. (**A-H**) The organs from four *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice were collected, sectioned and stained. H&E (A′-H′) and β-galactosidase (A″-H″) staining patterns in brain (A), heart (B), skin (C), kidney (D), lung (E) thymus (F), spleen (G) and testes (H). (**I**) Western blot showing the expression level of knockin HA-Cdk2 (upper panel) and endogenous Cdk2 (bottom panel) in various adult organs collected from *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice. Scale bars: 100 μm, except 250 μm in H′,H″.

genetic replacement of Cdk1 by Cdk2 leads to early embryonic lethality, similar to *Cdk1* deletion, and embryos die before E3.5. This indicates that Cdk1 is essential for the initial divisions that lead to the formation of the blastocyst. In addition, deletion of *p53* in the knockin background did not rescue the phenotypes caused by the substitution of Cdk1 by Cdk2. From this genetic replacement study, we were only able to obtain heterozygous knockin mice (*Cdk1*$^{+/Cdk2KI}$), in which one copy of *Cdk2* is expressed from the *Cdk1* locus and the other allele encodes wild-type *Cdk1*. Our work indicates that at least one copy of *Cdk1* is essential for the survival of mice and that Cdk2 cannot substitute for Cdk1 function, even when expressed from the *Cdk1* locus. Among the possible reasons for the failed rescue is that the localization of Cdk2KI differs from that of Cdk1, although differences in substrate specificity cannot be excluded either.

Moreover, when we crossed *Cdk1*$^{+/Cdk2KI}$ with *Cdk2*$^{+/-}$ mice and analyzed *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice, we found that both male and female *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice were sterile, similar to *Cdk2*$^{-/-}$ mice, even though they express Cdk2 from the *Cdk1* locus. When we analyzed the transcriptional activation of the *Cdk1* locus and the presence of knockin Cdk2 in testes by *lacZ* expression (β-galactosidase staining), HA western blotting and HA immunohistochemistry, we found abundant expression of knockin Cdk2 in the testes of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ mice. However, in adult *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice, testes and ovaries were atrophic, similar to *Cdk2*$^{-/-}$ mice. In *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice, we observed that the seminiferous tubules were smaller than normal with a substantial depletion of germ cells, similar to *Cdk2*$^{-/-}$ mice. As a result of the substantial depletion of germ cells, the expression of knockin Cdk2 was diminished in the seminiferous tubules. Furthermore, knockin Cdk2 was expressed similarly in germ cells of *Cdk2*$^{+/+}$ *Cdk1*$^{+/Cdk2KI}$ and *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ P10 testes when the germ cells were not developed beyond tetraploid primary spermatocytes. By contrast, atrophic testes lacked round spermatids and displayed extensive apoptosis of germ cells in *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ mice at P20, indicating that although knockin Cdk2 was expressed, it was unable to complete the pachytene stage and the cells instead underwent apoptosis.

We observed that *Cdk2*$^{-/-}$ spermatocytes arrested and accumulated mostly prior to pachytene. This arrest appears to be incomplete, as we observed occasional cells with pachytene morphology. We believe that this arrest can be overcome by knockin Cdk2, as we see more cells with pachytene morphology in *Cdk2*$^{-/-}$ *Cdk1*$^{+/Cdk2KI}$ than in *Cdk2*$^{-/-}$ mice. It appears, however, that this rescue is only partial, as these spermatocytes arrest later in pachytene. Given that the subcellular localization of knockin Cdk2 appears to reflect that of the endogenous Cdk2, we conclude that the timing of expression of Cdk2 is crucial for its meiotic function(s). These results suggest the existence of a certain time window for the requirement of Cdk2. When Cdk2 is not expressed at that particular time point, the cells fail to complete meiosis even though Cdk2 is

expressed subsequently, as indicated by the continuous HA and Cdk2 staining in the tubules of the $Cdk2^{-/-}$ $Cdk1^{+/Cdk2KI}$ mice after pachytene. Our results indicate that the genetic relocation of *Cdk2* to the *Cdk1* locus abolished Cdk2 meiotic function and as a result $Cdk2^{-/-}$ $Cdk1^{+/Cdk2KI}$ mice are sterile, similar to $Cdk2^{-/-}$ mice. This indicates that the genetic locus and timing of *Cdk2* expression determine the meiotic functions of Cdk2.

When we analyzed the subcellular localization of knockin Cdk2, we found that it was predominantly localized in the nucleus irrespective of the cell cycle stage, similar to endogenous Cdk2 (Moore et al., 1999). Although expressed from the *Cdk1* locus, knockin Cdk2 retains its subcellular localization. This indicates that the genomic locus does not play a significant role in determining the translocational property of a protein, at least in the case of Cdk2. Similarly, we found that knockin Cdk2 is able to form a complex with cyclin E1 and cyclin A2 and displays kinase activity similar to endogenous Cdk2. This excludes the possibility that the presence of the HA tag affected the properties, and thereby meiotic function, of knockin Cdk2. In addition, when we analyzed the proliferation rate of $Cdk2^{-/-}$ $Cdk1^{+/Cdk2KI}$ MEFs, we did not observe any significant difference to $Cdk2^{+/+}$ $Cdk1^{+/Cdk2KI}$ MEFs. This indicates that the knockin Cdk2 is able to perform its function in the mitotic cell cycle and form a complex with cyclin E1. In addition, analysis of cell cycle initiation and progression in vivo revealed that there was no significant difference between $Cdk2^{-/-}$ $Cdk1^{+/Cdk2KI}$ and $Cdk2^{+/+}$ $Cdk1^{+/Cdk2KI}$ mice, indicating that knockin Cdk2 was able to rescue the slight S-phase delay originally identified in $Cdk2^{-/-}$ mice during liver regeneration. Furthermore, we analyzed the transcriptional activation of the *Cdk1* locus by *lacZ* reporter gene expression using liver regeneration as an in vivo cell cycle model. This analysis revealed that *Cdk1* transcriptional activation occurred earlier in the absence of Cdk2, suggesting that premature activation of *Cdk1* is essential in the absence of Cdk2 in order to promote the G1–S transition. This observation is in accordance with our recent finding that Cdk1, as judged by protein level, is induced at an earlier time point in the absence of Cdk2 [(Satyanarayana et al., 2008), see Fig. 5C therein]. It appears that premature transcriptional and translational activation of *Cdk1* are essential in the absence of Cdk2 to drive cells through the G1–S transition by binding to cyclin E. In this context, it will be interesting to determine which molecular mechanisms are responsible for coordinating the transcriptional activation of *Cdk1* and *Cdk2*. When we analyzed the transcriptional activation of *Cdk1* in adult tissues by *lacZ* expression, we did not observe β-galactosidase staining in most of the tissues. This might be due to the fact that most of the adult organs are quiescent and mitotically inactive. By contrast, we found solid transcriptional activation of *Cdk1* during different stages (E14.5 to E20.5) of embryogenesis. Our results suggest that Cdk1 is essential for the differentiation and development of various organs during embryogenesis.

The present study indicates that Cdk1 is essential for the survival of mice. Genetic substitution of Cdk1 by Cdk2 leads to early embryonic lethality. This indicates that Cdk2 cannot substitute for the loss of Cdk1, even when the timing of transcription and genetic location of *Cdk2* match those of *Cdk1*. Most interestingly, Cdk2 loses its meiotic function when expressed from the *Cdk1* locus, even though it is able to perform its mitotic cell cycle functions by complexing with cyclin E1 and cyclin A2. In addition, an increase in the transcriptional activation of *Cdk1* during late embryogenesis (E14.5 to E20.5) indicated that Cdk1 is not only essential for early embryogenesis, but might also be essential in the latter stages of embryogenesis.

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Supplementary material

Supplementary material for this article is available at
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