



CDK5RAP2 Is Required to Maintain the Germ Cell Pool during Embryonic Development

Sami Zaqout,^{1,2,3} Paraskevi Bessa,¹ Nadine Krämer,^{1,2,3} Gisela Stoltenburg-Didinger,¹ and Angela M. Kaindle^{1,2,3,*}

¹Institute of Cell Biology and Neurobiology, Charité – Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany

²Center for Chronically Sick Children (Sozialpädiatrisches Zentrum, SPZ), Charité – Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany

³Berlin Institute of Health (BIH), Kapelle-Ufer 2, 10117 Berlin, Germany

*Correspondence: angela.kaindle@charite.de

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SUMMARY

Gene products linked to microcephaly have been studied foremost for their role in brain development, while their function in the development of other organs has been largely neglected. Here, we report the critical role of *Cdk5rap2* in maintaining the germ cell pool during embryonic development. We highlight that infertility in *Cdk5rap2* mutant mice is secondary to a lack of spermatogenic cells in adult mice as a result of an early developmental defect in the germ cells through mitotic delay, prolonged cell cycle, and apoptosis.

INTRODUCTION

Biallelic mutations in the cyclin-dependent kinase-5 regulatory subunit-associated protein 2 gene *CDK5RAP2* cause autosomal recessive primary microcephaly type 3 (MCPH3), a rare disease characterized by severe congenital microcephaly and intellectual disability (Bond et al., 2005; Kaindle et al., 2010; Kraemer et al., 2011; Moynihan et al., 2000). MCPH is acknowledged as a model disorder for an isolated brain phenotype. Recent data link the brain phenotype to a stem cell defect with premature shift from symmetric to asymmetric progenitor cell divisions, leading to premature neurogenesis, a depletion of the progenitor pool, and a reduction of the final number of neurons (Buchman et al., 2010; Fish et al., 2006; Kaindle et al., 2010; Lizarraga et al., 2010). In addition, reduced propagation and survival of differentiating neural progenitors have been shown (Kraemer et al., 2015).

Despite the highlighted brain phenotype, it needs to be noted that *Cdk5rap2* is ubiquitously expressed (Issa et al., 2013) and exerts functions such as maintaining centrosome function, spindle assembly and orientation, and/or cell cycle checkpoint control (Kraemer et al., 2011; Megraw et al., 2011) that are likely relevant also to other organs. So far, no progeny of affected humans has been reported, indicating a potential role of *CDK5RAP2* for the germline. Moreover, a loss of the *CDK5RAP2* homologous gene centrosomin (*cnm*) in *Drosophila* causes malfunctions in meiotic centrosomes and spermatid basal bodies leading to male sterility (Li et al., 1998). *Cdk5rap2* mutant or Hertwig's anemia (*an/an*) mice were known solely for their hematopoietic phenotype (macrocytic, hypoproliferative anemia, leukopenia) prior to their identification as an MCPH3 model with microcephaly in 2010 (Lizarraga

et al., 2010). Homozygous male *Cdk5rap2* mutant mice are infertile secondary to a severe germ cell deficiency, and females cannot deliver pups (Lizarraga et al., 2010; Russell et al., 1985). Here, we show that germ cell depletion in *an/an* mice occurs already during early development through a mitotic delay, prolonged cell cycle, and apoptosis.

RESULTS AND DISCUSSION

Hypomorphic Gross Phenotype and Embryonic Lethality

The *an/an* mice can be recognized by their characteristic hypomorphic gross phenotype apparent at birth (Figures 1A and 1A'). While the expected Mendelian ratio of *an/an* mice was found at embryonic days E12.5–E14.5 (*an/an*: *an/+*: *+/+* = 24: 43: 16), only 9.5% of the offspring carried a homozygous mutant genotype (*an/an*) at P0 (*an/an*: *an/+*: *+/+* = 46: 287: 152; Figure S1), indicating in utero lethality.

Sterility in Male Mice

Testes of *an/an* mice at both P0 and adult ages were severely reduced in cross-sectional area, weight, and testes/body weight ratio (Figures 1B–1F'). Further analysis of H&E-stained testes revealed the absence of gonocytes in P0 *an/an* testes and of all spermatogenic cells from spermatogonia to mature sperms in adult *an/an* testes (Figures 1G and 1G'). We further confirmed this by immunostaining, applying germ cell markers anti-mouse vasa homolog (MVH) and anti-germ cell-specific antigen (TRA98) (Figure 2A and data not shown). The seminiferous tubules, demarked by Sertoli cells, were normal in architecture, but notably

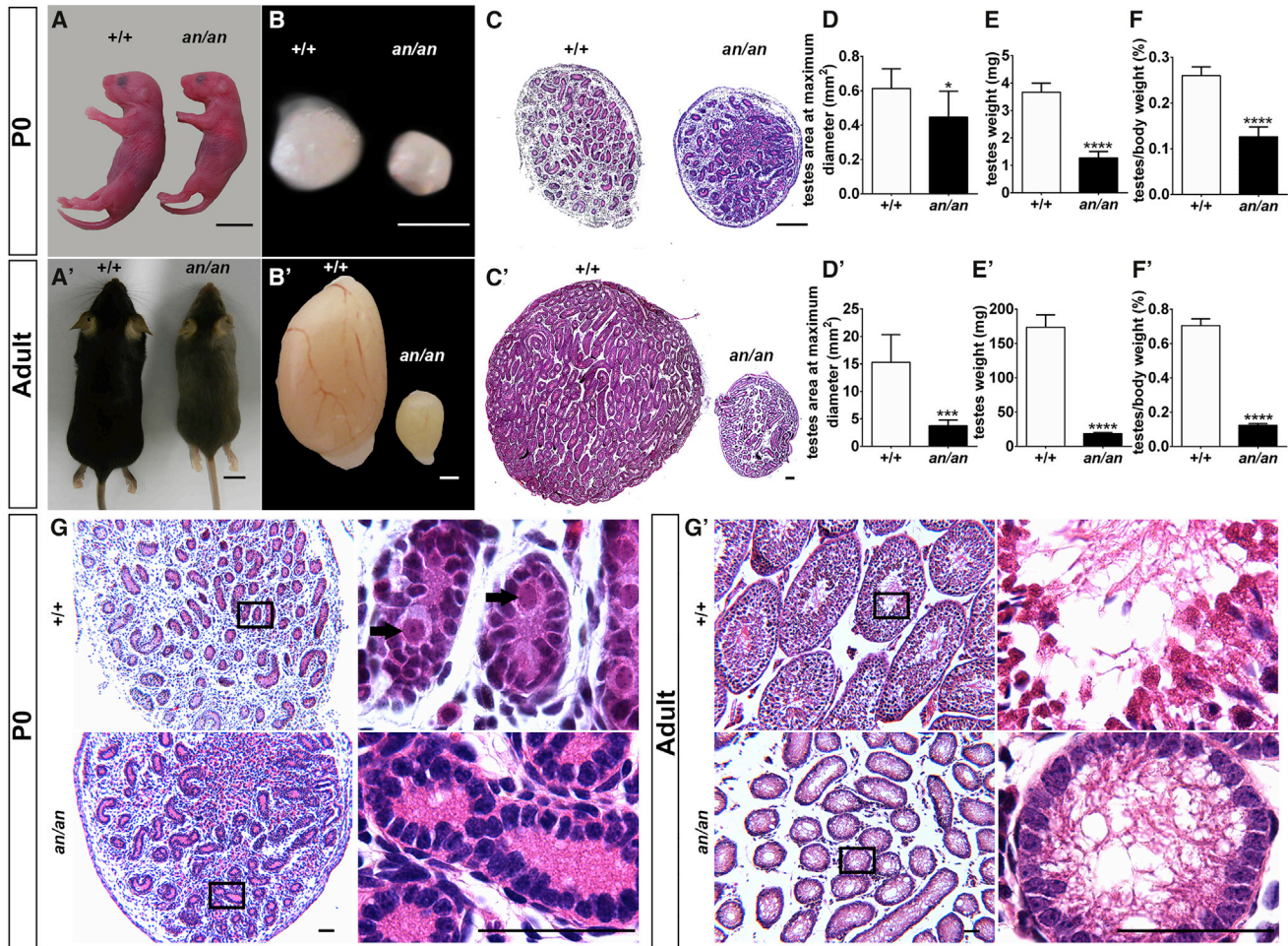


Figure 1. Testes of *Cdk5rap2* Mutant Mice Lack Germ Cells

(A–B') Hypomorphic gross phenotype and reduced testis size of P0 and adult *an/an* mice. Scale bars, 10 mm (A and A') and 1 mm (B and B'). (C–D') Testis area is reduced in P0 and adult *an/an* mice at the position of maximal testis diameter. Scale bars, 200 μ m, n = 6–7 animals/group.

(E–F') Reduction of testis weight and testis/body weight is already present at P0 in *an/an* mice and also significant in adult mice. n = 3–6 (P0) and n = 6–7 (adult) animals/group.

(G–G') Absence of gonocytes (arrows) in seminiferous tubules at P0 and of spermatogenic cells in adult *an/an* mice. Scale bars, 50 μ m, H&E staining, differential interference contrast images.

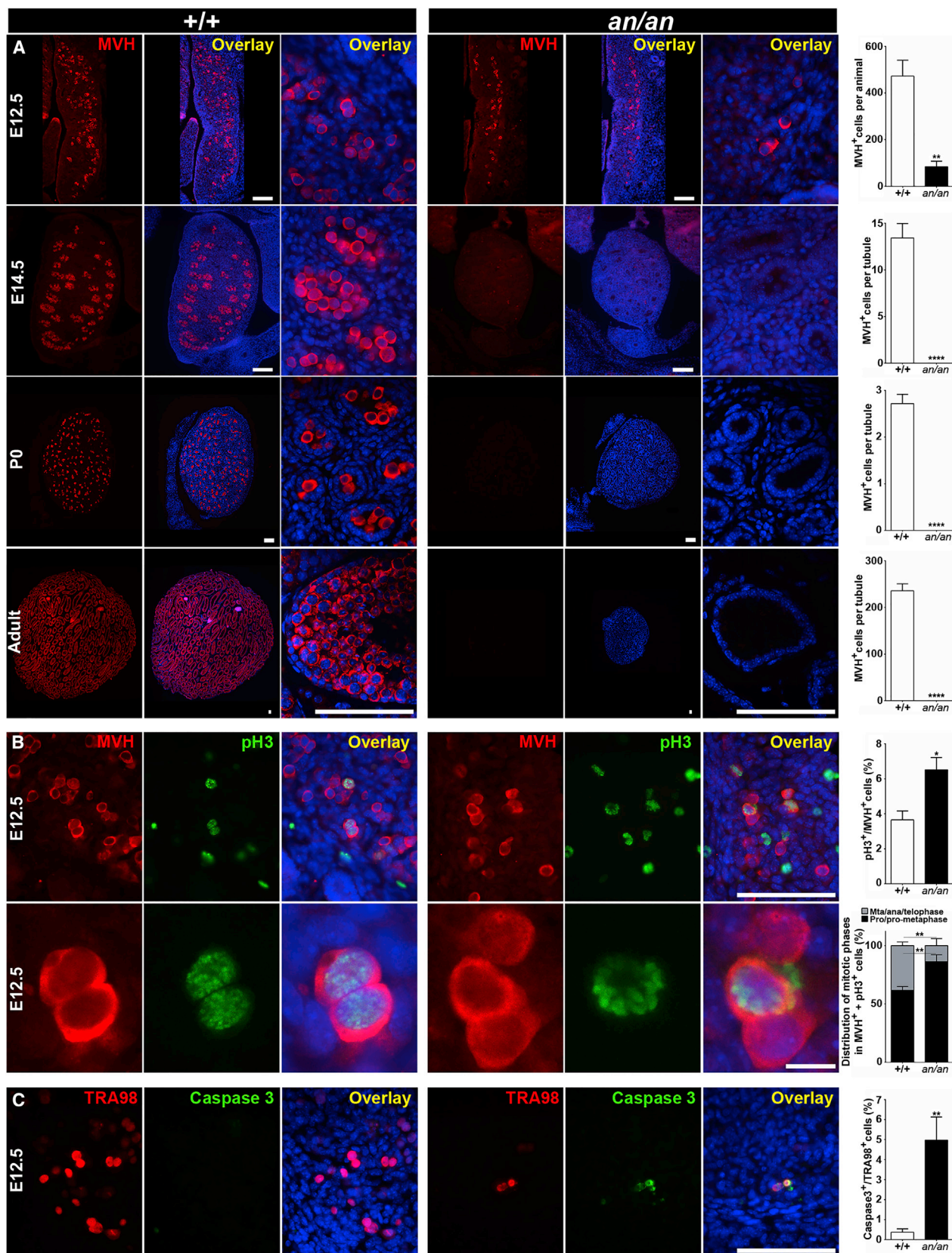
Error bars indicate SD, Student's t test, *p < 0.05, ***p < 0.001, ****p < 0.0001.

smaller in size in *an/an* mice due to lack of the germ cells. The Sertoli and Leydig cells appear normal, suggesting that these testicular somatic cells play no major role in *an/an* germ cell phenotype. Intriguingly, neither uterus nor ovaries could be detected in adult *an/an* females (Figure S2).

Lack of Germ Cells in *an/an* Mice Is due to an Early Developmental Defect

The absence of germ cells in *an/an* males at P0 points toward an earlier developmental defect in the germline. Normally, germ cells in mice are specified at E6.25–7.25, proliferate and migrate toward the genital ridge at E8.5–12.5, and

undergo mitotic arrest at E12.5–E14.5 in males (De Felici, 2009; McLaren, 2003; Western et al., 2008). The current availability of germ cell markers and the advantage of immunohistochemistry techniques allowed us to explore the exact time period when these cells are lost in *an/an* mice during development. For this, we studied embryonic sections of *an/an* mice at the level of the testes and the genital ridges at E14.5 and E12.5, respectively. At E14.5, testes of *an/an* mice lacked gonocytes positive for MVH or TRA98 (Figure 2A and data not shown). Compared with wild-type +/+ mice, E12.5 *an/an* mouse sections showed a



(legend on next page)



significantly reduced number of gonocytes/primordial germ cells in the genital ridge (Figure 2A). We could not detect any aberrant located cells, i.e., cells outside the vicinity of the genital ridges. Thus, abnormal migration is unlikely the cause of germ cell depletion in *an/an* mice.

To further investigate the fate of the germ cells in *an/an* mice, we studied their proliferation and apoptosis behavior at E12.5. Here, more germ cells were positive for mitotic cell marker phospho-histone H3 (pH3) in *an/an* compared with *+/+* mice (Figure 2B). Further analysis of these mitotic cells revealed more pro/pro-metaphase cells in *an/an* compared with *+/+* mice (Figure 2B). Using activated caspase-3 as an apoptotic cell marker, we found a significant increase in apoptotic germ cells in *an/an* compared with *+/+* mice (Figure 2C). The increase in the relative number of mitotic germ cells in E12.5 *an/an* mice with more cells in pro/pro-metaphase and a lower total number of germ cells indicate a delay in mitotic progression of these cells. Intriguingly, loss of *Cdk5rap2* mutant germ cells in *an/an* mice occurs at a time when these cells physiologically exit the cell cycle and enter a mitotic quiescent phase.

Cdk5rap2 Is Required for Normal Germ Cell Cycle Progression

We then asked whether germ cell cycle progression is affected in *an/an* mice. To answer this, we performed successive pulse labeling of heterozygous (*an/+*) pregnant mice at E11.5 with two different thymidine analogs: 5-iodo-2'-deoxyuridine (IdU) and 5-bromo-2'-deoxyuridine (BrdU, interval 4 hr). Thirty minutes after the second pulse, the E11.5 embryos were collected and double immunostained with rat anti-BrdU (which detects both IdU- and BrdU-labeled cells) and mouse anti-BrdU (which specifically detects BrdU-labeled cells) (Figure 3A). This enabled us to estimate the ratio of germ cells that left the cell cycle within a time frame of 4 hr. In E11.5 *+/+* embryos, we found that many germ cells already exited the cell cycle after 4 hr, as detected by exclusive positivity for IdU ($54.49\% \pm 3.2\%$). In *an/an* embryos, however, less germ cells had exited the cell cycle during the same time frame ($39.02\% \pm 1.8\%$, $p < 0.01$) (Figure 3).

Our results suggest increased apoptosis as a consequence of mitotic delay and prolonged cell cycle as the likely cause

of *Cdk5rap2* mutant germ cell depletion (Figure 4). This is in line with recent studies linking mitotic delay to increased apoptosis of neuronal progeny (Pilaz et al., 2016) as well as evidence stressing apoptosis as a critical regulator of the germ cell pool (Aitken et al., 2011). In agreement with a germ cell defect in the MCPH3 mouse model, a severe reduction in testis volume due to a massive loss of germ cells has been reported in a mouse model of MCPH5 (Pulvers et al., 2010). The underlying pathomechanism may be similar to that reported here in the MCPH3 mouse model. Our findings highlight the critical role of *Cdk5rap2* in maintaining the germ cell pool during embryonic development in mice. Compared with centrosomin mutant *Drosophila* (Li et al., 1998), the stronger phenotype and the different mechanism in the mouse vertebrate model may reflect that *Cdk5rap2* has gained additional functions during evolution. We report prolonged mitosis of germ cells parallel to (and putatively leading to) massive apoptosis in the progeny as a cause of infertility. Understanding the cause leading to infertility is likewise important for understanding the fate of neural progenitors and thus mechanisms leading to microcephaly in MCPH.

EXPERIMENTAL PROCEDURES

Mice

Cdk5rap2 mutant or Hertwig's anemia mice (*an/an*) carrying an inversion of exon 4 (that leads to exon skipping [Lizarraga et al., 2010]) were generated by crossing heterozygous (*an/+*) mice (C57BL/6 background; Jackson Laboratory, stock no. 002306). The breeding was performed during the day at the animal facility of the Charité – Universitätsmedizin Berlin, Germany. The adult mice included in this study were P53-P79. Genotyping was performed using the PCR primers for (*+/+*) F 5'-TC ACT GAG CTG AAG AAG GAG AA-3', R 5'-TGT CTT TCT GCC CTG ACA GT-3' and (*an/an*) F 5'-GC AAT CAC TAA AAT GTC CGA TT-3', R 5'-TGT CTT TCT GCC CTG ACA GT-3' with an expected 1,047 bp band in *+/+*, a 500 bp band in *an/an*, and both bands in (*an/+*). All experiments were carried out in accordance with the national ethics principles (registration no. T0309.09).

Histology and Immunofluorescence Staining

After dissection, the tunica albuginea of adult testes was punctured with a needle to allow rapid penetration of the fixative (4%

Figure 2. Germ Cells in *Cdk5rap2* Mutant Mice Are Lost by E14.5

(A) MVH-positive germ cells are reduced in number in the genital ridge of E12.5 *an/an* mice and are absent by E14.5. Scale bars, 100 μ m. (B) Increase in the relative number of mitotic germ cells (MVH and pH3 double-positive cells) in *an/an* mice with more cells in pro/pro metaphase. Scale bars, 100 μ m (upper panel) and 10 μ m (lower panel); average of 472 germ cells counted per *+/+* animal and 113 germ cells counted per *an/an* animal. (C) Increase in the relative number of apoptotic germ cells (TRA98 and activated caspase-3 double-positive cells) in *an/an* mice. Scale bars, 100 μ m; average of 246 germ cells counted per *+/+* animal and 96 germ cells counted per *an/an* animal. Immunofluorescence images, $n = 4-5$ (E12.5), $n = 6$ (E14.5), $n = 5-6$ (P0), and $n = 6-7$ (adult) animals/group. Error bars indicate SEM, Student's *t* test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

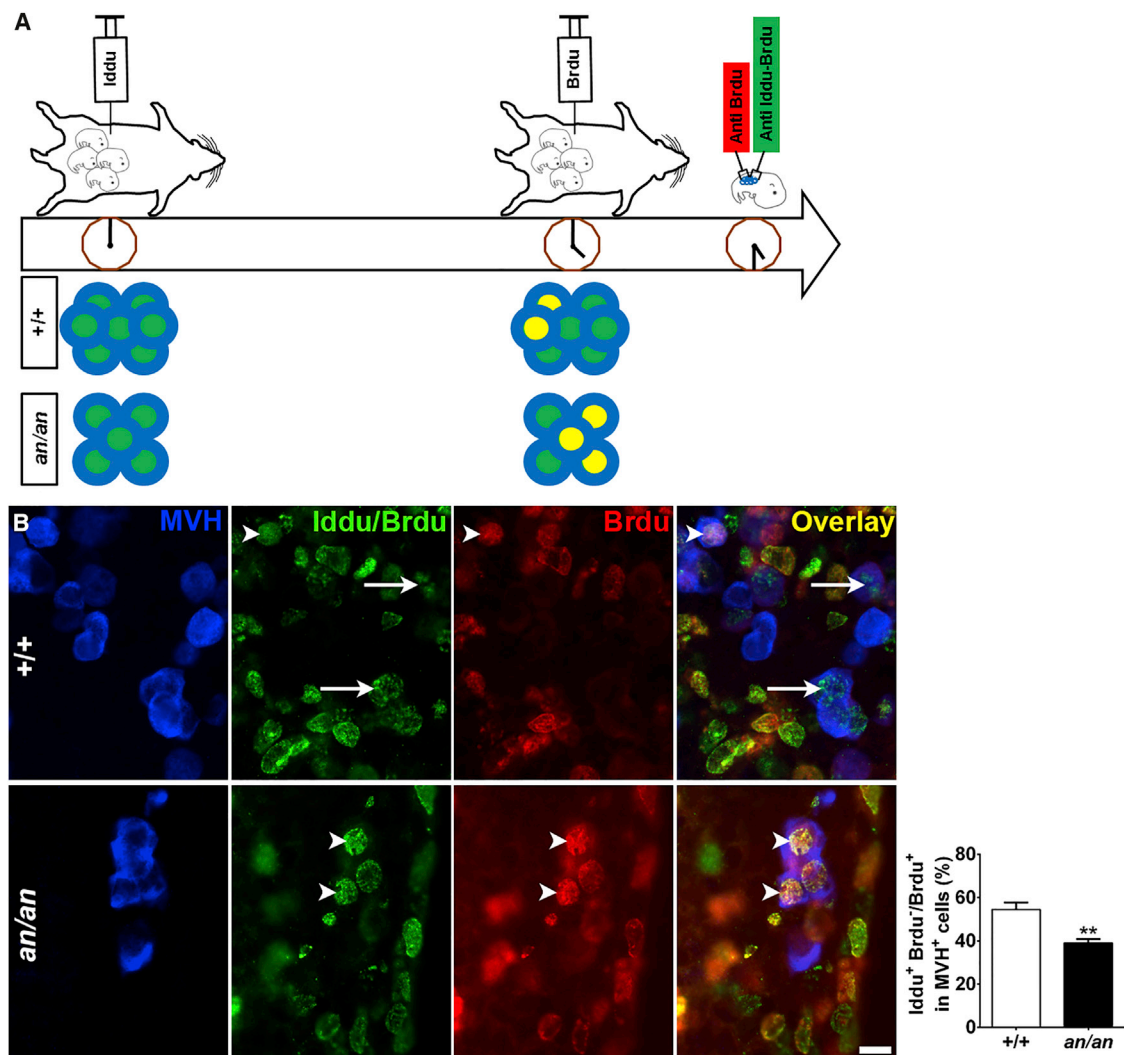


Figure 3. Cdk5rap2 Is Required for Normal Germ Cell Cycle Progression

(A) Protocol used to study cell cycle progression at E11.5 in vivo. Successive pulse labeling of heterozygous (*an/+*) pregnant mice at E11.5 with two different thymidine analogs to detect cycling cells.

(B) Decrease in the relative number of exclusively IddU-positive germ cells which left the cell cycle in *an/an* mice; green-filled circles in (A) and arrows in (B). Dual-labeled cycling cells are indicated by yellow-filled circles in (A) and arrowheads in (B). Scale bar, 10 μ m; immunofluorescence images, average of 647 germ cells counted per *+/+* animal and 182 germ cells counted per *an/an* animal, *n* = 5 animals/group. Error bars indicate SEM, Student's *t* test, ***p* < 0.01.

paraformaldehyde in 0.12 M TPO₄ phosphate buffer). At E12.5 and E14.5, whole embryos were used. Following fixation for 10 min (P0), 4 hr (adult), or overnight (E12.5, E14.5), testes and embryos were dehydrated in an ethanol series (50%, 70%, 85%, 90%, and 100%), cleaned with xylene, and embedded in paraffin. Sections of 5 μ m were cut on a microtome and collected on Superfrost Plus slides.

P0 and adult testes sections at the position of maximal testis diameter were deparaffinized and stained with H&E. For immunostaining, deparaffinized sections were exposed to a heat-mediated antigen retrieval step with citrate-based solution (H-3300, pH 6.0), blocked in 10% donkey or goat normal serum for 1 hr at room temperature (RT) prior to incubation with the primary antibodies over-

night in a humid chamber at RT. Sections were then incubated with the corresponding secondary antibodies for 2 hr at RT, followed by PBS 1 \times rinsing and treatment with a solution containing 10 mM CuSO₄ and 50 mM NH₄Cl (pH 5) to reduce autofluorescence. Finally, the sections were washed with dd-H₂O and mounted with Immumount. Primary antibodies were applied as follows: rabbit anti-MVH/DDX4 (1:500; Abcam, ab13840), rat anti-germ cell-specific antigen TRA98 (1:500; Abcam, ab82527), mouse anti-phospho-histone H3 (1:100; Cell Signaling Technology, 9706), rabbit anti-activated cleaved caspase-3 (1:200; Cell Signaling Technology, 9661), mouse anti-BrdU (1:300; Millipore, MAB3424, clone AH4H7-1), and rat anti-BrdU (1:250; Abcam, ab6326). Secondary antibodies

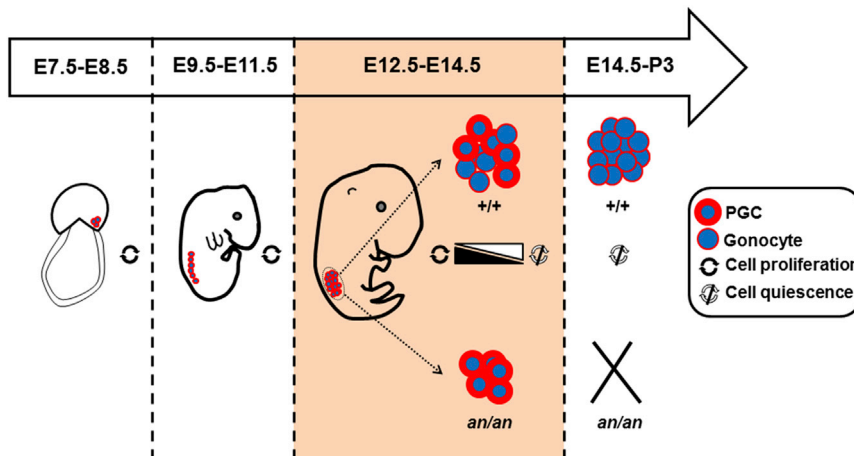


Figure 4. Schematic Model of Mechanism Leading to Embryonic Loss of Germ Cells in *Cdk5rap2* Mutant Mice

Germ cells are specified at around E7.5, proliferate, and migrate toward the genital ridge around E8.5–12.5 in mice. In *an/an* mice, germ cells undergo massive apoptosis at a time when the cells undergo physiologically mitotic arrest (E12.5–E14.5). This results in a loss of germ cells during embryonic development, which is apparent later in male infertility.

(1:400) were goat Alexa Fluor 488 conjugate anti-mouse immunoglobulin G (IgG), goat Alexa Fluor 488 conjugate anti-rabbit IgG, goat Cy3-conjugate anti-rat IgG (Invitrogen), and donkey Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch). Nuclei were stained with DAPI (1:1000, Sigma-Aldrich).

Imaging

Bright-field images were taken with an Olympus BX60 microscope with an AxioCam MRc Zeiss camera and AxioVision 4.8 software (Zeiss). The fluorescent images were taken by an Olympus BX51 microscope with an Intas camera and MagnaFire 2.1B software (Olympus). All images were processed using Adobe Photoshop CS6 version 13.0x64.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2017.01.002>.

AUTHOR CONTRIBUTIONS

A.M.K. and S.Z. were responsible for the project conception and wrote the manuscript. S.Z., P.B., N.K., and G.S. performed the experiments. All authors read, revised, and approved the final manuscript.

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Supplemental Information

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Supplementary figures

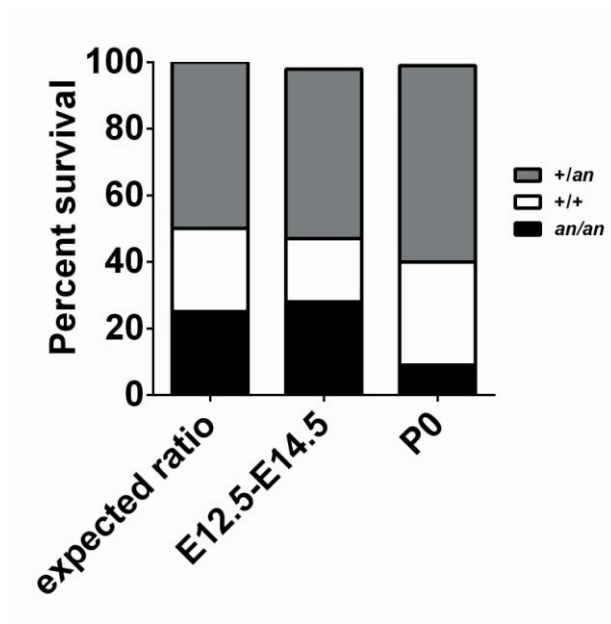


Figure S1. Partial embryonic lethality of *Cdk5rap2* mutant mice. Expected proportion of *an/an* mice were found at E12.5-E14.5, but only 9.5% of the offspring were *an/an* at P0, underlining partial embryonic lethality of mutant mice.



Figure S2. Genital phenotype in *Cdk5rap2* mutant females. Uterus (white arrow) in the *+/+* and ovaries are absent in *an/an* females.