

# The Alpha Catalytic Subunit of Protein Kinase CK2 Is Required for Mouse Embryonic Development<sup>∇</sup>

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**Protein kinase CK2 (formerly casein kinase II) is a highly conserved and ubiquitous serine/threonine kinase that is composed of two catalytic subunits (CK2 $\alpha$  and/or CK2 $\alpha'$ ) and two CK2 $\beta$  regulatory subunits. CK2 has many substrates in cells, and key roles in yeast cell physiology have been uncovered by introducing subunit mutations. Gene-targeting experiments have demonstrated that in mice, the CK2 $\beta$  gene is required for early embryonic development, while the CK2 $\alpha'$  subunit appears to be essential only for normal spermatogenesis. We have used homologous recombination to disrupt the CK2 $\alpha$  gene in the mouse germ line. Embryos lacking CK2 $\alpha$  have a marked reduction in CK2 activity in spite of the presence of the CK2 $\alpha'$  subunit. CK2 $\alpha^{-/-}$  embryos die in mid-gestation, with abnormalities including open neural tubes and reductions in the branchial arches. Defects in the formation of the heart lead to hydrops fetalis and are likely the cause of embryonic lethality. Thus, CK2 $\alpha$  appears to play an essential and uncompensated role in mammalian development.**

CK2 is a ubiquitous and highly conserved serine/threonine kinase. With hundreds of putative substrates identified through *in vitro* studies (27), CK2 has been linked to fundamental cellular processes including the regulation of DNA transcription and damage responses (13, 14), protein translation and stability (5, 42), and circadian rhythms (22). Many studies have detected dysregulated expression of CK2 in human cancers (46). CK2 was found to be upregulated in lymphocytic pseudo-leukemia in cattle infected by the parasite *Theileria parva* (32). We modeled this in transgenic mice that developed lymphoma with the overexpression of CK2 $\alpha$  alone (40) or synergistically in collaboration with *c-myc*, *Tal-1* overexpression (17), or *p53* loss (19). We have found CK2 $\alpha$  to be highly expressed in human and rodent mammary tumors, and transgenic expression of CK2 $\alpha$  can also promote breast cancer in mice (20). These experiments validate the powerful role of CK2 in cellular growth control. However, to identify the essential functions of CK2, we and others have used gene targeting by homologous recombination.

CK2 genes were first deleted in yeast by homologous recombination. While mammals have two homologous catalytic subunits, CK2 $\alpha$  and CK2 $\alpha'$ , and a single regulatory subunit, CK2 $\beta$ , the fission yeast *Schizosaccharomyces pombe* has single catalytic and regulatory subunits. *Orb5* is a mutant yeast strain in the catalytic CK2A1 subunit, and these yeasts have defects in polarized cell growth (41). Disruption of the regulatory CK2B1 subunit in *S. pombe* produces a cold-sensitive phenotype and abnormalities in cell shape (36). *Saccharomyces cerevisiae* has two catalytic and two regulatory subunits; temperature-sensitive alleles of CK2A1 show defects in cell polarity (35), and temperature-sensitive alleles of CK2A2 have defects in cell cycle progression with a dual-arrest phenotype at both the G<sub>1</sub>

and G<sub>2</sub>/M transitions (16). Deletion of CK2B1 leads to a salt-sensitive phenotype, indicating that the enzyme in yeast has a role in ion homeostasis (2). A genomic screen of the CK2 yeast mutants shows the dysregulation of hundreds of genes (1).

In mice, deletion of the single CK2 $\beta$  regulatory subunit by homologous recombination is early embryonic lethal in a cell-autonomous fashion (4). Precisely what fundamental cell process was blocked is unclear, because those investigators were unable to generate CK2 $\beta^{-/-}$  embryonic fibroblasts or embryonic stem (ES) cells. In contrast, we found that the deletion of the minor CK2 $\alpha'$  catalytic subunit is well tolerated, as homozygous null CK2 $\alpha'^{-/-}$  mice are viable but the males are infertile (51). The developing spermatocytes are frequently defective and undergo apoptosis, leading to oligospermia; the surviving spermatozoa are abnormal and resemble those seen in the human infertility syndromes of “globozoospermia” (round-headed sperm). We have now targeted the more abundant CK2 $\alpha$  subunit by homologous recombination. Mice lacking CK2 $\alpha$  die in mid-gestation, with structural defects in heart and neural tube, highlighting the specific role of CK2 $\alpha$  in the development of these organs.

## MATERIALS AND METHODS

**CK2 $\alpha$ -targeting construct.** Long-range PCR was used to amplify a total of 8.3 kb of genomic DNA from the 129SvEv BAC clone pBeLoBAC11-240o17 (Incyte Genomics) as 3.7- and 4.6-kb arms. Each arm was then sequentially cloned into pBluescriptII KS(+) and pPNT (23) targeting vectors. The 5' arm (3.7 kb) spanned the translational start site to the second coding exon, ending upstream of the codon for lysine 68 (K68). The 3' arm (4.6 kb) began 85 nucleotides downstream of the 5' arm and spanned the next three exons, thereby deleting the K68 residue critical for ATP binding (Fig. 1A). The 5' arm was amplified using the following PCR primers containing KpnI restriction sites: forward primer 5'-AAGGTACCAAGCAGGGCCAGAGTTTACA-3' and reverse primer 5'-AAGGTACCAACTGTATT TGCCCTACCTAA-3'. The 3' arm was amplified using the following PCR primers with restriction sites (XhoI on the forward primer and SacI and NotI on the reverse primer): forward primer 5'-GGGATCTCGAGAGTTACTTGGAATGTAGAGT-3' and reverse primer 5'-AATGAGCTCGCGCCGCTTAATTACA GTTCTATTCG-3'. Final concentrations of PCR components were as follows: 200  $\mu$ M each deoxynucleoside triphosphate, 400 nM each primer, 1.8 mM Mg<sup>2+</sup>, 1 U eLONGase enzyme mix (In-

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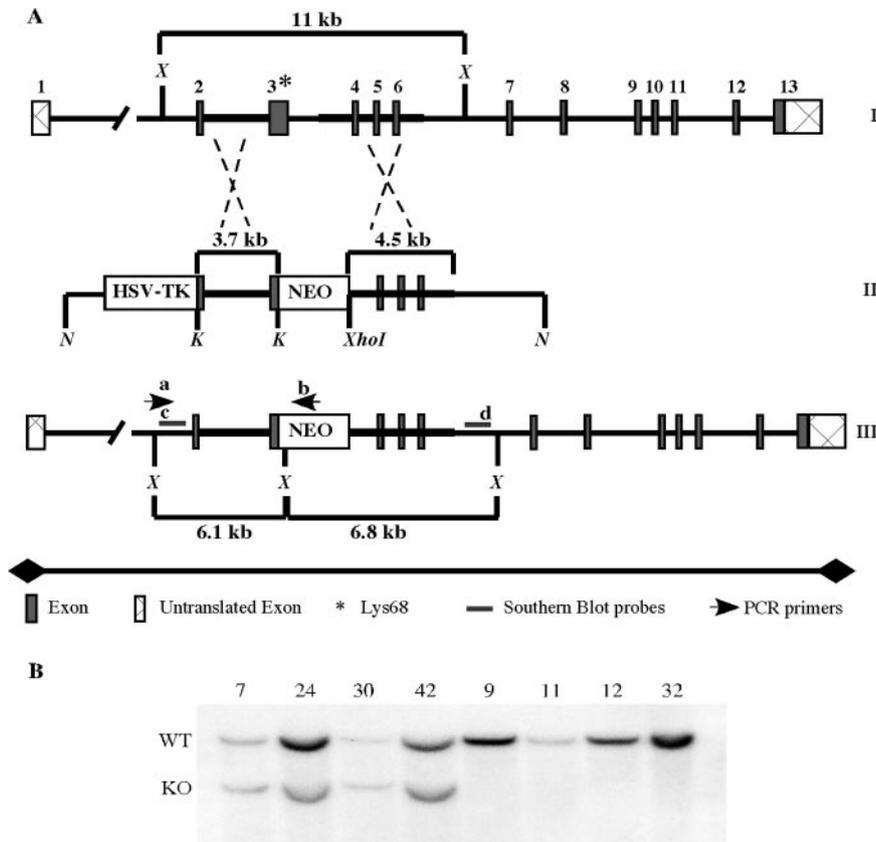


FIG. 1. CK2 $\alpha$  targeting. (A) CK2 $\alpha$ -targeting vector (II) and targeted allele (III). To generate a CK2 $\alpha$  null allele, an 85-bp region of the gene (I) (numbers indicate exons) that included the critical K68 residue (\*) in the catalytic ATP-binding pocket was targeted. A 3.7-kb 5'-flanking arm and 4.5-kb 3'-flanking arm were cloned into vector pPNT using KpnI (K), NotI (N), and XhoI. Screening was carried out by PCR using an external flanking and internal *neo* PCR primer pair (a and b, respectively). (B) To confirm homologous recombination, Southern blotting was carried out following XmnI (X) restriction digestion. A 5'-flanking probe (c) was used to identify the 11-kb wild-type (WT) and 6.1-kb homologously recombined DNA fragments, confirmed with a 3' probe (d) (not shown). ES cell colonies 7, 24, 30, and 42 were hemizygous for the targeted CK2 $\alpha$  allele, while colonies 9, 11, 12, and 32 were wild type. HSV, herpes simplex virus; KO, knockout.

vitrogen), and 500 ng DNA template. Thermocycling was performed for 35 cycles with denaturation at 94°C and annealing and extension at 68°C. Each flanking arm was individually cloned into pBluescriptII KS(+) (Stratagene) using KpnI for the 5' arm and XhoI and SacI for the 3' arm. The final CK2 $\alpha$ -targeting construct was assembled by subcloning the 5' and 3' arms into pPNT using KpnI, XhoI, and NotI and confirmed by restriction mapping and PCR.

**ES cell transfection, selection, and identification of homologous recombinants.** TC1 ES cells, kindly provided by Philip Leder, were cultured in ES medium consisting of Dulbecco's modified eagle's medium (Cellgro) supplemented with 12.5% ES-grade fetal bovine serum (Sigma), 2% L-glutamine, 1% nonessential amino acids, 1% penicillin, and 1% streptomycin supplemented with  $5 \times 10^5$  U Esgro-Lif (Chemicon) on mitomycin-treated mouse embryonic fibroblast feeder cells prepared from a neomycin-resistant mouse in a 7.5% CO<sub>2</sub> incubator at 37°C. ES cells ( $1 \times 10^7$  cells) were electroporated with 40  $\mu$ g (1  $\mu$ g/ $\mu$ l) of NotI-linearized targeting vector at 250 V and 250  $\mu$ F, resuspended in 10 ml of ES medium, and evenly split into 10 10-cm tissue culture plates containing inactivated fibroblast feeder cells with ES medium without selection. Drug selection with 260  $\mu$ g/ml G418 (Gibco) was started 24 h following transfection for 48 h, followed by the addition of 0.1  $\mu$ M 1-(2-deoxy-2-fluoro- $\beta$ -D-arabinofuranosyl)-5-iodouracil (FIAU) for an additional 96 h. Fresh drug-containing medium was changed daily. Undifferentiated drug-resistant ES colonies were picked when they reached the appropriate size and expanded for freezing and DNA analysis. ES cell DNA was prepared using the Qiagen DNeasy tissue kit according to the manufacturer's instructions. Homologous recombination was confirmed by PCR and Southern blotting. For the PCR, a 5' primer upstream of the targeting arm, 5'-CK2aKO\_F (5'-CCAACGTCT GCTTTTGAACA-3'), and a 3' primer in the neomycin cassette, CK2aKO\_R2 (5'-TCGCCT TCTTGACG AGTTCT-3'), were used. The PCR product was 4,357 bp in size and identified

the CK2 $\alpha$  knockout allele. The presence of the homologously recombined allele was confirmed by Southern blotting using [<sup>32</sup>P]dCTP-labeled 5' and 3' DNA probes. Probes were radiolabeled using a hexanucleotide random priming mix (Invitrogen) and Klenow (NEB) fragment according to the manufacturers' protocols. For Southern blots, 20  $\mu$ g of genomic DNA from tail DNA or ES cells was digested with XmnI, separated on 1% agarose gels, and transferred in 400 mM NaOH onto a Gene Screen Plus (Perkin-Elmer) by capillary flow. Hybridization with labeled probes was performed with QuikHyb hybridization solution (Stratagene) according to the manufacturer's protocol and exposed to Kodak film for 24 to 72 h.

**Generation and genotyping of knockout mice.** All animal experimentation was performed with the approval of the Boston University Medical Center IACUC. Mice were initially maintained in a two-way specific-pathogen-free barrier facility in microisolator cages; subsequent generations were transferred to a one-way facility. Targeted ES cell clones were microinjected into C57BL/6 blastocysts in the microinjection facility at Tufts-New England Medical Center. In all, 52 injected blastocysts were transplanted into three pseudopregnant recipient female mice, resulting in 18 chimeric mice. High-grade male agouti coat chimeras were identified and bred with wild-type C57BL/6 females to test for germ line transmission of the targeted CK2 $\alpha$  allele. F<sub>1</sub> agouti offspring were screened by PCR and Southern blotting using tail DNA prepared by proteinase K digestion and high-salt extraction. Identification of CK2 $\alpha$ <sup>+/-</sup> F<sub>1</sub> mice confirmed germ line transmission of the targeted CK2 $\alpha$  allele; heterozygous F<sub>1</sub> mice were intercrossed to attempt to generate homozygous CK2 $\alpha$  knockout mice in the F<sub>2</sub> generation.

**Embryo isolation and genotyping.** For timed matings, heterozygous CK2 $\alpha$ <sup>+/-</sup> mice were interbred, and the females were checked the next morning for vaginal plugs, which was estimated to be 0.5 days postconception and therefore equiv-

alent to embryonic day 0.5 (E0.5). Pregnant females were maintained in their breeding cages for the appropriate number of days and then sacrificed for embryo collection. Embryos were removed from the uterine horns and processed immediately: they were frozen for protein analysis, homogenized in TRIzol reagent (Gibco BRL) for RNA extraction, or fixed in fresh 4% glutaraldehyde for histology. Embryonic genomic DNA was extracted from yolk sacs using the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. Genotyping was performed using a three-primer PCR consisting of one forward primer, CK2aKO\_del\_F (5'-CCACCATGTCTGGCATTAAA-3'), and two reverse primers, CK2aKO\_del\_R (5'-TTCCCTCTTTGACCACATC-3') and CK2aKO\_R2 (5'-TCGCCTTCTTGACGAGTTCT). Primers CK2aKO\_del\_F and CK2aKO\_del\_R amplified a 406-bp product only from the wild-type allele, and primers CK2aKO\_del\_F and CK2aKO\_R2 amplified a 650-bp product only from the targeted allele. Thirty-five cycles of thermocycling were performed, with denaturation at 94°C, annealing at 57°C, and extension at 72°C.

**Analysis of embryonic gene expression.** Embryo RNA was pretreated with DNase I to digest contaminating genomic DNA. First-strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). For reverse transcription (RT)-PCR, the following primer sets were used: 5'-ATCAAGGAAGGCTTTA GCAAATGGG-3' and 5'-GAACCTCGATTACATCGTGAGA-3', amplifying a 159-bp product, for brachyury (50); 5'-CGGTGTCCAACACAGATCT G-3' and 5'-TCTCTCGAGTGGGTTGAC-3', amplifying a 187-bp product, for ANF (50); 5'-TGAGGGAGAGCGCAGGCTCAAG-3' and 5'-TGCTGTC CACGATGGACGTAAGG-3', amplifying a 361-bp product, for myogenin (50); 5'-GCCAAGAAGCGGATAGAAGG-3' and 5'-TGTGGTTCAGGGCTCAGT C-3', amplifying a 499-bp product, for MLC-2V (28); and 5'-CAGACCTGAA GGAGACCT-3' and 5'-GTCAGCGTAAACAGTTGC-3', amplifying a 286-bp product, for MLC-2A (18). The following thermocycler conditions were used for brachyury, myogenin, and ANF: 4 min of initial denaturation at 95°C; 31, 33, or 35 cycles of amplification (25 s at 94°C, 30 s at 60°C, and 45 s at 72°C); a 6-min final extension at 72°C; and a hold at 4°C. The following thermocycler conditions were used for MLC-2A and MLC-2V: 4 min of initial denaturation at 95°C; 30, 32, or 34 cycles of amplification (30 s at 94°C, 30 s at 50°C, and 45 s at 72°C); a 10-min final extension at 72°C; and a hold at 4°C.

**Histology and in situ hybridization.** Histologic and in situ hybridization techniques were previously described (45, 51). Briefly, freshly dissected whole embryos were fixed for 2 h with 4.3% glutaraldehyde followed by overnight fixation with 1% osmium tetroxide, dehydrated, and embedded in plastic. Sections (1 to 2  $\mu$ m) were cut and either deplasticized and stained with hematoxylin and eosin or stained with toluidine blue. For in situ hybridization, embryos were fixed in fresh 4% paraformaldehyde in phosphate-buffered saline overnight at 4°C. Following dehydration and paraffin embedding, 5- $\mu$ m sections were cut, rehydrated, and hybridized with antisense riboprobes to *Csnk2a1*, *Csnk2a2*, and *Csnk2b* that had been radiolabeled with [<sup>35</sup>S]UTP (51). Following hybridization, slides were exposed, developed, fixed, and photographed. Adjacent sections were stained with hematoxylin and eosin.

**CK2 protein expression and activity.** For immunoblotting and kinase assays, E10.5 embryos were washed in ice-cold phosphate-buffered saline and rapidly transferred to lysis buffer containing 40 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 125 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and Sigma protease inhibitor cocktail, followed by centrifugation to remove debris. Yolk sacs were used for genotyping as described above. Protein was quantified by BCA assay (Pierce), and 15  $\mu$ g was separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membrane (Millipore). Monoclonal antibody for CK2 $\alpha$ /CK2 $\alpha'$  was obtained from BD Biosciences, that for CK2 $\beta$  was obtained from Calbiochem, and that for actin was AC-15, from Sigma. Goat anti-mouse horseradish peroxidase-conjugated secondary antibody was obtained from Santa Cruz Biotechnology. Visualization was performed using ECL (Pierce). For CK2 kinase activity, 7.5  $\mu$ g of protein was incubated with 0.1 mM CK2-specific peptide substrate RRREEE TEEE (Sigma-Genosys Inc.) in CK2 kinase buffer (100 mM Tris [pH 8.0], 20 mM MgCl<sub>2</sub>, 100 mM KCl, 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]GTP) at 30°C for 20 min. Control reactions were carried out without peptide. The reaction was stopped by adding 10 mM ATP in 0.4 N HCl. Samples were spotted onto P81 Whatmann filters and washed in 150 mM H<sub>3</sub>PO<sub>4</sub> to remove unincorporated [ $\gamma$ -<sup>32</sup>P]GTP, and phosphorylated peptides were measured by scintillation counting. Samples were assayed in duplicate, and background kinase activity in the absence of the peptide substrate was subtracted. *P* values were assessed by analysis of variance; Bonferroni correction was applied for multiple comparisons.

TABLE 1. Yield of live births from CK2 $\alpha$ <sup>+/-</sup> × CK2 $\alpha$ <sup>+/-</sup> matings<sup>a</sup>

Genotype	Expected frequency if viable	Expected frequency if lethal	No. of animals	Observed frequency
CK2 $\alpha$ <sup>+/+</sup>	0.25	0.33	80	0.33
CK2 $\alpha$ <sup>+/-</sup>	0.5	0.67	163	0.67
CK2 $\alpha$ <sup>-/-</sup>	0.25	0	0	0

## RESULTS

**CK2 $\alpha$  gene targeting.** We generated mice in which the CK2 $\alpha$  gene was disrupted by replacing the exon encoding the critical ATP-binding residue, lysine 68 (30), with a neomycin resistance cassette. The targeting construct, pPNT-CK2 $\alpha$ KO (Fig. 1A), was linearized by NotI digestion and electroporated into TC1 ES cells. Transfected ES cells were subjected to positive and negative selection in G418 and FIAU, and colonies that survived double selection were screened by PCR for homologous recombination. Four out of the first 40 ES colonies screened were identified as being potential homologous recombinants (results not shown). This result was confirmed by Southern blotting (Fig. 1B). Clone 7 was chosen for injection into C57BL/6 embryos; high-grade chimeras were obtained and mated to wild-type C57BL/6 females. The presence of agouti F<sub>1</sub> offspring demonstrated that targeted ES cells contributed to the germ line, and the positive identification of CK2 $\alpha$ <sup>+/-</sup> F<sub>1</sub> mice by both PCR and Southern blotting confirmed germ line transmission of the knockout allele (not shown).

**CK2 $\alpha$ <sup>-/-</sup> embryos are nonviable.** All of the CK2 $\alpha$ <sup>+/-</sup> F<sub>1</sub> mice were viable, indistinguishable from their CK2 $\alpha$ <sup>+/+</sup> littermates, and fertile. CK2 $\alpha$ <sup>+/-</sup> mice were intercrossed to generate F<sub>2</sub> progeny. These progeny were screened by PCR and Southern blotting for the presence of the homologously recombined allele. Out of 243 F<sub>2</sub> mice genotyped from more than 30 litters, no CK2 $\alpha$ <sup>-/-</sup> mice were recovered at weaning (Table 1). The ratio of CK2 $\alpha$ <sup>+/+</sup> to CK2 $\alpha$ <sup>+/-</sup> pups at weaning was 1:2, consistent with the expected frequency for an embryonic lethal phenotype of CK2 $\alpha$ <sup>-/-</sup> mice, with no selection against CK2 $\alpha$ <sup>+/-</sup> births.

**CK2 $\alpha$ <sup>-/-</sup> embryos lack CK2 $\alpha$  transcript and protein and have diminished CK2 kinase activity.** To determine whether the CK2 $\alpha$ -null allele generated mRNA transcript and protein products, embryos (CK2 $\alpha$ <sup>+/+</sup>, CK2 $\alpha$ <sup>+/-</sup>, and CK2 $\alpha$ <sup>-/-</sup> littermates) were collected at E10.5. Genotyping of each embryo was performed by PCR of genomic DNA extracted from the corresponding yolk sacs. CK2 $\alpha$ <sup>-/-</sup> embryos were pooled, and mRNA was prepared for amplification by RT-PCR. No CK2 $\alpha$  mRNA transcripts were detected in the CK2 $\alpha$ <sup>-/-</sup> embryos (Fig. 2A). Protein analysis performed on individual embryos indicated that the CK2 $\alpha$ <sup>-/-</sup> embryos lacked any detectable CK2 $\alpha$  protein (42 kDa) but had amounts of CK2 $\alpha'$  protein (38 kDa) that were similar to those of their littermates, indicating that there was no compensatory upregulation of CK2 $\alpha'$  (Fig. 2B). However, CK2 $\beta$  levels were reduced in the knockouts (data not shown). The CK2 $\alpha$ <sup>+/-</sup> embryos consistently expressed approximately half the amount of CK2 $\alpha$  protein compared to their CK2 $\alpha$ <sup>+/+</sup> littermates (Fig. 2B). CK2 kinase activity was measured using a specific CK2 peptide substrate

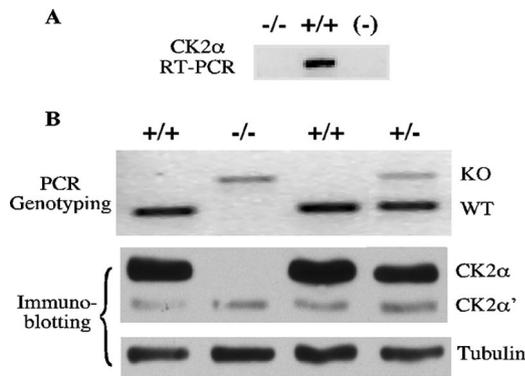


FIG. 2. CK2 $\alpha^{-/-}$  embryos lack CK2 $\alpha$  mRNA and protein. (A) To detect CK2 $\alpha$  mRNA transcripts, E10.5 embryos were genotyped individually by PCR using DNA prepared from the yolk sacs. Three CK2 $\alpha^{-/-}$  ( $-/-$ ) and three CK2 $\alpha^{+/+}$  ( $+/+$ ) embryos were pooled, and RT-PCR was performed; - indicates CK2 $\alpha^{+/+}$  RNA without RT. (B) To assay for CK2 $\alpha$  protein, E10.5 embryos were genotyped, and protein extracts were prepared. Fifteen micrograms of protein from individual embryos was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted using an anti-CK2 $\alpha$ /CK2 $\alpha'$  antibody. Wild-type CK2 $\alpha^{+/+}$  ( $+/+$ ) embryos contained a major immunoreactive band at  $\sim$ 42 kDa, consistent with CK2 $\alpha$ , and a weaker band at  $\sim$ 38 kDa, consistent with CK $\alpha'$ . In the knockout (KO) CK2 $\alpha^{-/-}$  ( $-/-$ ) embryos, the intensity of the CK2 $\alpha'$  band was unchanged, and the CK2 $\alpha$  band was absent. Heterozygote CK2 $\alpha^{+/-}$  ( $+/-$ ) embryos reproducibly had a CK2 $\alpha$  band of half the intensity of the wild type (WT). Tubulin was used as a loading control.

and [ $\gamma$ - $^{32}$ P]GTP as a phosphate donor. Activity in the CK2 $\alpha^{+/-}$  embryos was 68% of that of the CK2 $\alpha^{+/+}$  embryos ( $P = 0.0002$ ), and in the CK2 $\alpha^{-/-}$  embryos, it was 23% ( $P = 0.0001$ ).

**Developmental defects in CK2 $\alpha^{-/-}$  embryos.** Timed matings were performed to determine when the CK2 $\alpha^{-/-}$  embryos die. A total of 155 embryos including 27 CK2 $\alpha^{-/-}$  embryos were examined. Throughout development, the CK2 $\alpha^{+/-}$  embryos were indistinguishable from CK2 $\alpha^{+/+}$  embryos. Beginning at E8.5, developmental abnormalities were observed in CK2 $\alpha^{-/-}$  embryos (Fig. 3A and B). While the CK2 $\alpha^{-/-}$  embryos had normal anteroposterior length at E8.5, their primitive hearts were enlarged in comparison to those of CK2 $\alpha^{+/+}$  embryos, and their neural folds were convex, while the neural folds of the CK2 $\alpha^{+/+}$  embryos had elevated and begun to fuse (Fig. 4A).

By E9.5, the abnormalities in the CK2 $\alpha^{-/-}$  embryos became more apparent (Fig. 3C and D). At this stage, blood is normally seen circulating in the yolk sac and the embryo, indicating that hematopoiesis has begun and that the primitive heart tube is circulating blood, and we observed this in the CK2 $\alpha^{+/+}$  and CK2 $\alpha^{+/-}$  embryos. However, even though the CK2 $\alpha^{-/-}$  hearts were contracting, they were enlarged in relation to the size of the embryo and appeared distended. Defects in the neural tube and head region of CK2 $\alpha^{-/-}$  embryos were variable in severity; typically, the rostral end was closed, but closure of the cranial portion failed to occur (Fig. 4B), while in a few embryos, the entire neural tube was open (craniorachischisis) (Fig. 4C). The head shape was abnormal, and the forebrain, midbrain, and hindbrain regions of CK2 $\alpha^{-/-}$  embryos were consistently smaller than those of their wild-type counterparts; there

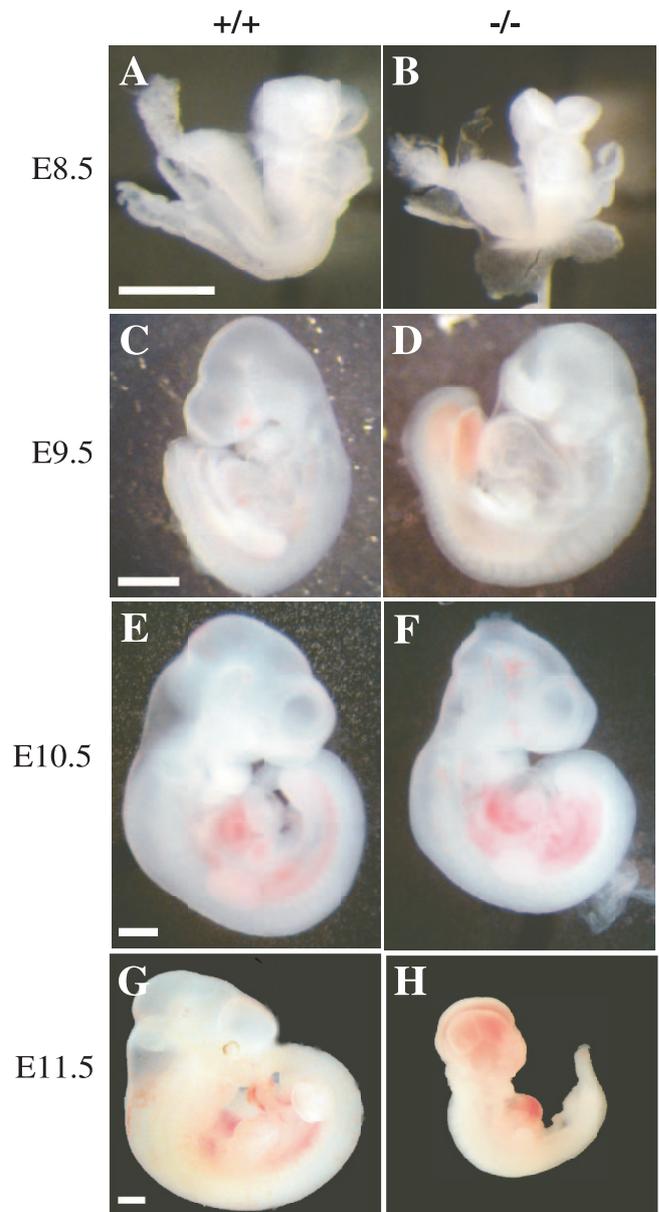


FIG. 3. Comparison of wild-type CK2 $\alpha^{+/+}$  (A, C, E, and G) and knockout CK2 $\alpha^{-/-}$  (B, D, F, and H) embryos from E8.5 to E11.5; white scale bars (500  $\mu$ m) indicate the relative sizes of embryos between developmental stages. At E8.5 (A and B), the neural folds in the CK2 $\alpha^{-/-}$  embryos failed to elevate like those of CK2 $\alpha^{+/+}$  littermates. By E9.5 (C and D), CK2 $\alpha^{-/-}$  hearts were larger than those of their littermates; CK2 $\alpha^{-/-}$  neural tubes failed to fuse at the cranial level. At E10.5 (E and F), CK2 $\alpha^{-/-}$  embryos were usually smaller, hearts were dilated, and head shape was abnormal. At E11.5 (G and H), CK2 $\alpha^{-/-}$  embryos were runted, hearts were no longer beating, and blood could be seen hemorrhaging into the cranium and thorax.

was no expansion of the telencephalic vesicles. The eye fields were identifiable but indistinct and underdeveloped in the CK2 $\alpha^{-/-}$  embryos, and the second branchial arch was usually underdeveloped (Fig. 4D to F and Table 2). Limb bud differentiation was delayed in CK2 $\alpha^{-/-}$  embryos compared with wild-type embryos. The tailbud of the CK2 $\alpha^{-/-}$  embryos was short and terminated in a broad, blunt end instead of a tapered

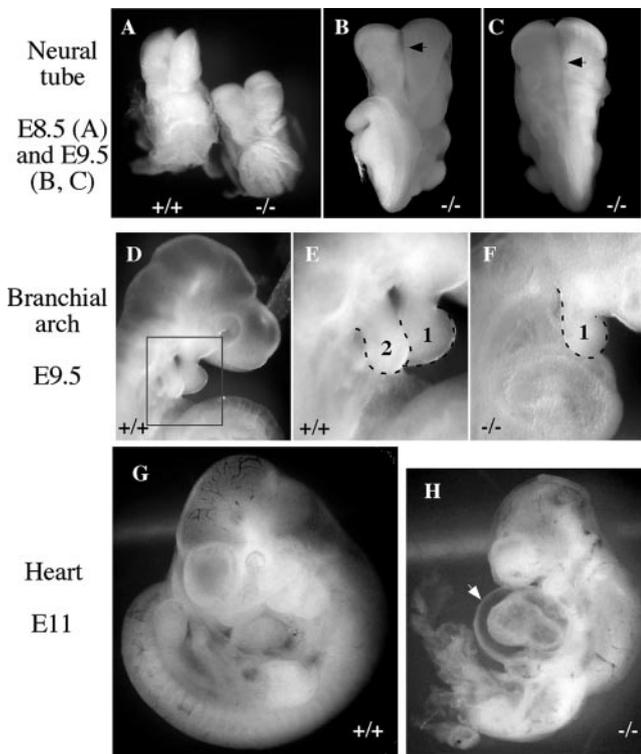


FIG. 4. Details of developmental defects in CK2 $\alpha^{-/-}$  embryos. (A to C) At E8.5, CK2 $\alpha^{-/-}$  neural folds failed to elevate (A). At E9.5, the neural folds failed to fuse along the anterior-posterior axis (arrows indicate the unfused neural tube on the ventral side [B] or dorsal side [C]). (D to F) By E9.5, wild-type embryos have developed the second branchial arch (D) (inset is enlarged in panel E), but it was smaller in most of the CK2 $\alpha^{-/-}$  embryos (F) (Table 2). (G and H) At E11, the pericardial sacs of the CK2 $\alpha^{-/-}$  embryos were edematous (arrow) due to heart failure, i.e., hydrops fetalis.

end as in the wild-type. Because of the cranial and tailbud defects, the CK2 $\alpha^{-/-}$  embryos were reduced in their antero-posterior lengths.

By E10.5, the CK2 $\alpha^{-/-}$  embryos were significantly smaller than littermate controls (Fig. 3E and F) when matched for somite number. Their hearts were contracting, and blood was circulating, but hearts were enlarged (E11) (Fig. 4G and H). Pericardial effusions, a sign of high-output cardiac failure, were sometimes seen in CK2 $\alpha^{-/-}$  embryos. The heads of CK2 $\alpha^{-/-}$  embryos remained smaller and malformed; the neural tubes remained open in this region, and the eye fields were underdeveloped, but the otic placodes were present. At matched developmental stages, the numbers of branchial arches were reduced in the knockout embryos (Table 2).

The CK2 $\alpha^{-/-}$  embryos did not progress beyond E10.5, and at E11.5, CK2 $\alpha^{-/-}$  embryos were not much bigger than normal E10.5 embryos (Fig. 3G and H). The branchial arches did not fuse, and the mouth was not formed. The optic vesicles, lens placodes, and retinal pigmentation were visible in wild-type embryos but were less pronounced in some CK2 $\alpha^{-/-}$  embryos and virtually absent in others. Limb bud development was retarded in the CK2 $\alpha^{-/-}$  embryos and never progressed beyond the limb bud paddle. At this stage, the hearts were large and dilated and no longer beating. Some of the embryos hem-

orrhaged into the thorax, abdomen, and cranium. Beyond E11.5, only a few necrotic CK2 $\alpha^{-/-}$  embryos could be identified.

**Histological abnormalities in CK2 $\alpha^{-/-}$  embryos.** Histologic sections were examined to determine the morphological basis of the neural tube and cardiac abnormalities seen in the developing embryos. Representative cranial and thoracic sections of E10.5 embryos demonstrated that the CK2 $\alpha^{-/-}$  embryos had an open neural tube in the forebrain region, although it was closed but collapsed in the region of the hindbrain (Fig. 5A and B). The rudiments of the telencephalic vesicles in the forebrain did not expand outward, and the optic stalk and evagination of the optic vesicle were collapsed. The otic vesicles were rounder in the CK2 $\alpha^{-/-}$  embryos, lacking the dorsal extension seen in normal embryos, and the epithelium was thickened. The notochord, trigeminal ganglion, and facio-acoustic neural crest complex were present. Using both transmission electron microscopy and high-magnification light microscopy, mitoses were seen in both CK2 $\alpha^{+/+}$  and CK2 $\alpha^{-/-}$  embryos, indicating that the cells were viable and proliferating at this stage (not shown).

Transverse sections at the level of the thorax were also obtained in the same embryos. At this stage, the wild-type embryo was forming a four-chambered heart with trabeculation in the ventricles (Fig. 5C). In contrast, in CK2 $\alpha^{-/-}$  embryos, an open heart tube persisted, with an enlarged endomyocardial cavity with a thin and disorganized endothelial lining and reduced trabeculation and with a thin atrial wall. The surface ectoderm and presumptive parietal pericardial layer also appeared abnormal (Fig. 5D).

**Markers of differentiation in CK2 $\alpha^{-/-}$  embryos.** To begin to correlate molecular markers with the observed phenotypes in CK2 $\alpha^{-/-}$  embryos, we examined the expression of mRNA-encoding genes involved in mesoderm formation (brachyury and myogenin) and cardiac specification (MLC-2A, MLC-2V, and ANF) by semiquantitative RT-PCR (Fig. 6A). The number of amplification cycles was varied to ensure that the PCR was linear. Consistent with the phenotype of the mutant embryos, brachyury and myogenin were present in the CK2 $\alpha^{-/-}$  embryos, suggesting that gastrulation and the establishment of the mesoderm had taken place. Similarly, markers of heart development were present, suggesting that cardiomyocyte differentiation had begun in the CK2 $\alpha^{-/-}$  embryos. Hypoxanthine phosphoribosyltransferase was used as an internal control and demonstrated equal amounts of starting cDNA template between CK2 $\alpha^{-/-}$  and CK2 $\alpha^{+/+}$  samples (Fig. 6A).

**Expression pattern of CK2 mRNA transcripts in embryonic heart.** We examined the pattern of mRNA expression of the CK2 subunits in normal heart development using in situ hybridization (Fig. 6B). In situ hybridization for CK2 subunit

TABLE 2. Branchial arches in embryos at E10.5

Genotype	Total no. of embryos counted	% of embryos with number of branchial arches of:			
		4	3	2	1
CK2 $\alpha^{+/+}$	18	78	22	0	0
CK2 $\alpha^{+/-}$	16	69	19	13	0
CK2 $\alpha^{-/-}$	14	25	36	50	7

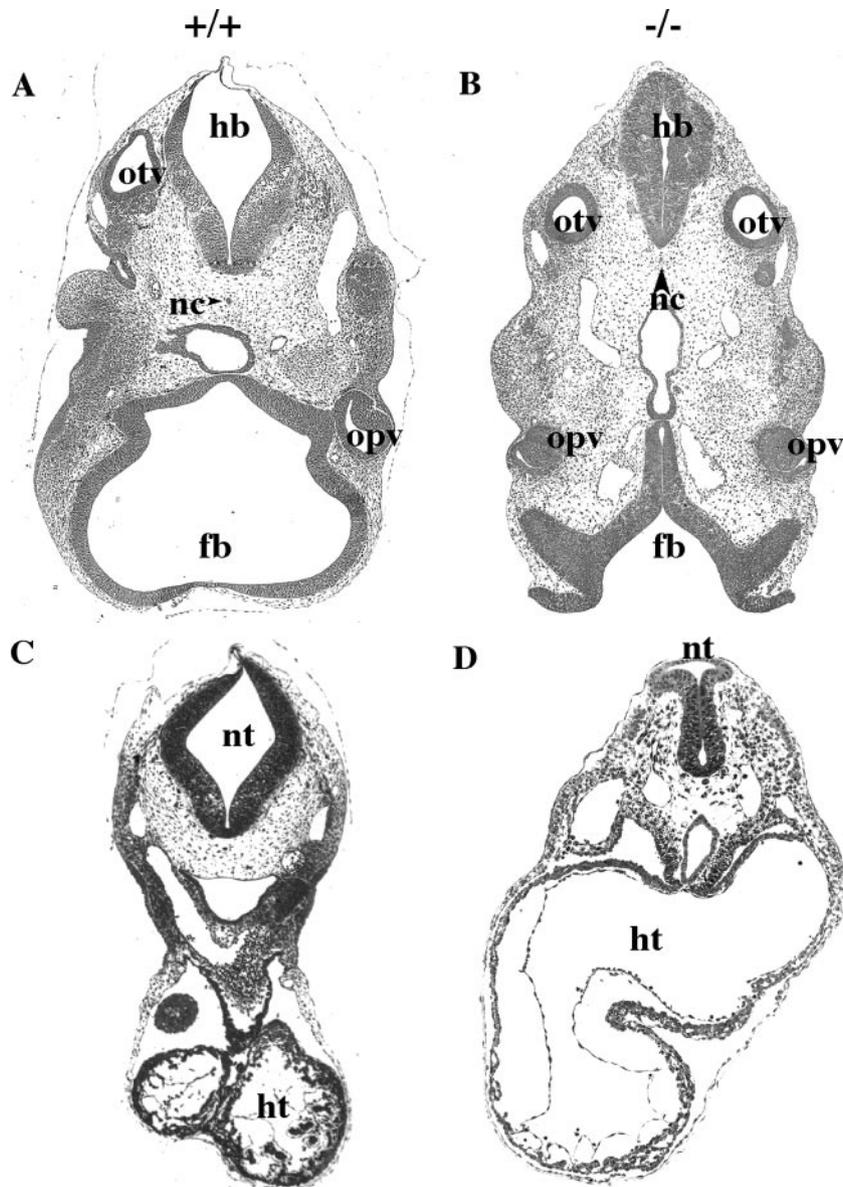


FIG. 5. Histological abnormalities in  $CK2\alpha^{-/-}$  embryos. Representative E10.5  $CK2\alpha^{+/+}$  and  $CK2\alpha^{-/-}$  embryos were fixed and sectioned through the cranial (A and B) and thoracic (C and D) regions. In the  $CK2\alpha^{+/+}$  head (A), the neural tube has fused, forming a sealed tube that is expanding in preparation for brain development. In the  $CK2\alpha^{-/-}$  embryo, because the neural tube (nt) is open anteriorly in the forebrain (fb) region, the posterior tube has collapsed, interfering with the normal development of the hindbrain (hb). The optic vesicles (opv) and the notochord (nc) appear to be similar, but the otic vesicles (oty) in the  $CK2\alpha^{-/-}$  embryo are round and thickened compared to those in the  $CK2\alpha^{+/+}$  embryo, which has a characteristic dorsal extension. (C and D) Whereas the  $CK2\alpha^{+/+}$  heart (ht) is forming chambers and developing trabeculation, the heart of the  $CK2\alpha^{-/-}$  embryo is still an open tube.

transcripts demonstrated strong relative expression of  $CK2\alpha$  mRNA in the ventricles at E13.5, while  $CK2\alpha'$  and  $CK2\beta$  appeared to have more uniform expression throughout the heart and the rest of the embryo.

#### DISCUSSION

We have demonstrated that  $CK2\alpha^{-/-}$  mice die in mid-embryogenesis, while  $CK2\alpha^{+/+}$  mice appear to be completely normal. The  $CK2\alpha^{-/-}$  embryos exhibited extensive cardiac and neural tube defects, and the embryonic lethality is likely

attributable to abnormalities in the structure and function of the heart. Embryonic hearts were defective in  $CK2\alpha^{-/-}$  embryos isolated from E8.5 to E10.5, although they were able to beat. Sections of the  $CK2\alpha^{-/-}$  hearts revealed defective formation of the chambers and poor trabeculation. The  $CK2\alpha^{-/-}$  embryos died at  $\sim$ E11, an age consistent with lethality due to defects in cardiac looping and chamber formation (6). Our RT-PCR results showed that transcript levels of the axial mesodermal marker brachyury and the striated muscle transcription factor myogenin were present but somewhat diminished in  $CK2\alpha^{-/-}$  embryos compared to wild-type embryos. In con-

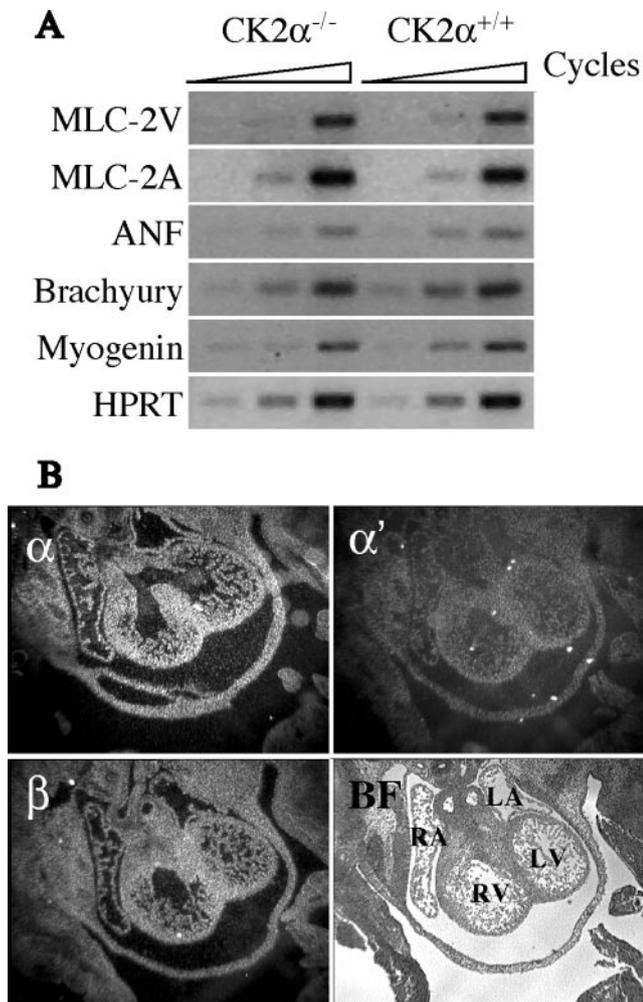


FIG. 6. mRNA expression in the embryos. (A) Semiquantitative RT-PCR was used to demonstrate the presence of mRNA encoding markers of mesoderm and cardiac formation in CK2 $\alpha^{-/-}$  and CK2 $\alpha^{+/+}$  embryos at E9.5. The triangles indicate increasing numbers of amplification cycles. Transcript levels of the cardiac markers MLC-2V, MLC-2A, and ANF and brachyury and myogenin, markers of mesoderm formation, were measured; hypoxanthine phosphoribosyltransferase (HPRT) was used as an mRNA control. (B) In situ hybridization for CK2 subunit transcripts demonstrates strong relative expression of CK2 $\alpha$  mRNA in the ventricles at E13.5, while CK2 $\alpha'$  and CK2 $\beta$  appear to have a more uniform expression throughout the embryo. Sense probes showed no signal and are omitted, and a bright-field (BF) image is shown for orientation, with the right atrium (RA), left atrium (LA), right ventricle (RV), and left ventricle (LV) labeled.

trast, transcripts for cardiac markers such as MLC-2A, MLC-2V, and ANF were unchanged. Thus, the defect in the CK2 $\alpha^{-/-}$  embryos is not due to a global defect in mRNA transcription.

The heart is the first organ to develop, and it is essential for embryonic development. The process of cardiogenesis is complex but begins with the specification of the cardiac mesoderm. The primitive heart tube develops from this specialized tissue and progresses through a tightly regulated series of morphological changes including looping of the heart tube, emergence of the endocardial cushion, and formation of the four chambers found in the adult. This process is controlled by the spa-

tiotemporal expression of a number of developmental pathways including the heregulin, bone morphogenetic protein, fibroblast growth factor, and Wnt pathways and transcription factors that include targets of these pathways as well as GATA, T-box, and Nkx proteins (33). Targeting of genes in these pathways typically results in abnormal cardiac development.

Similarly, the coordinate expression of multiple developmental pathways is required for normal neural tube closure (reviewed in reference 8), and pathways involved in this and subsequent brain development include sonic hedgehog signaling, Notch, and, again, the Wnt pathway. Neural tube defects are a common developmental abnormality in humans, including spina bifida, when the posterior neural tube fails to close; anencephaly, when the defect is anterior; or craniorachischisis, when the entire neural tube is open. All of these neural tube defects were observed in the CK2 $\alpha^{-/-}$  embryos. Closure of the neural tube allows the rapid expansion of brain volume due to fluid pressure exerted on the lumen of the closed neural tube (9, 10, 15, 34, 37, 38); when the neural tube fails to close, the neural tube collapses, as was seen in the CK2 $\alpha^{-/-}$  embryos. Neural tube defects, while severe, generally do not lead to embryonic lethality (7).

Thus, one of the pathways that is common to heart and brain development is the Wnt pathway (24–26, 31). The Wnt transcriptional cofactor  $\beta$ -catenin is required for normal heart formation (21), and the Wnt target *cripto* is required for the differentiation of cardiomyocytes, cardiogenesis, and neural tube formation (11, 29, 49, 50). Targeted deletion of *Wnt1* or *Wnt3a* causes defects in the midbrain and hindbrain regions and ectopic secondary neural tubes, respectively (24–26, 31, 44). *Dvl* knockouts have defects of closure of the neural folds (48). We have shown that CK2 is a critical regulator of Wnt signaling in cells and in *Xenopus laevis* embryos (12, 42, 43). Thus, the neural tube and heart defects in CK2 $\alpha^{-/-}$  embryos could be due, in part, to the dysregulation of the Wnt pathway. Because of the complex developmental regulation of the neural tube and heart, and the many cellular processes regulated by CK2, a precise determination of the mechanisms behind the developmental defects in the CK2 $\alpha^{-/-}$  embryos will require a thorough investigation of transcriptional and posttranslational regulation of components of Wnt and other signaling pathways using a variety of genomic and proteomic techniques.

In contrast to the essential role of CK2 $\alpha$  in embryogenesis, CK2 $\alpha'$  plays a required role in male germ cell development only (51). Thus, CK2 $\alpha$  and CK2 $\alpha'$  are not redundant. This may be due to the fact that CK2 $\alpha$  is the more abundant catalytic subunit in the developing embryo, accounting for more than three-fourths of the CK2 catalytic activity. Furthermore, the loss of CK2 $\alpha$  leads to diminished CK2 $\beta$  levels in the embryo, similar to what we observed previously with a reduction of CK2 $\alpha$  levels in cells using small interfering RNA oligonucleotides (39). Alternatively, the CK2 $\alpha$  and CK2 $\alpha'$  subunits may have functional differences; functional specialization of CK2 subunits has been seen in biochemical studies using dominant negative catalytic subunits (47) and through studies identifying unique partners (3). In the future, the issue of functional specialization could be resolved through knock-in experiments by substituting one catalytic subunit for another.

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