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While K-ras Is Essential for Mouse Development, Expression of the K-ras 4A Splice Variant Is Dispensable


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In mammals, the three classical ras genes encode four highly homologous proteins, N-Ras, H-Ras, and the isoforms K-Ras 4A and 4B. Previous studies have shown that K-ras is essential for mouse development and that while K-ras 4A and 4B are expressed during development, K-ras 4A expression is regulated temporally and spatially and occurs in adult kidney, intestine, stomach, and liver. In the present study, the pattern of K-ras 4A expression was examined in a wide range of wild-type adult mouse tissues, and gene targeting was used to generate K-ras 4A-deficient mice to examine its role in development. It was found that K-ras 4A is also expressed in uterus, lung, pancreas, salivary glands, seminal vesicles, bone marrow cells, and cecum, where it was the major K-Ras isoform expressed. Mating between K-ras<sup>tm4A/tm4B</sup> mice produced viable K-ras<sup>tm4A+</sup> offspring with the expected Mendelian ratios of inheritance, and these mice expressed the K-ras 4B splice variant only. K-ras<sup>tm4A/tm4B</sup> mice were fertile and showed no histopathological abnormalities on inbred (129/Ola) or crossbred (129/Ola × C57BL/6) genetic backgrounds. The results demonstrate that K-Ras 4A, like H- and N-Ras, is dispensable for normal mouse development, at least in the presence of functional K-Ras 4B.

The three classical mammalian ras genes, K-, N-, and H-ras, encode proteins that are members of the guanine nucleotide binding protein superfamily (8). Ras proteins are small (~21 kDa) GTPases which cycle between inactive (GDP-bound) and active (GTP-bound) conformations at the plasma membrane, by interaction with a variety of guanine nucleotide exchange factors and GTPase activating proteins in response to stimulation by a diverse array of cell surface receptors, including the epidermal growth factor receptor and cytokine receptors such as interleukin-2 (14). Following activation, Ras proteins bind and activate a plethora of downstream effector proteins, including Raf kinases and phosphatidylinositol 3-kinases, and by this means control many cellular functions, including proliferation, differentiation, migration, and apoptosis (4, 11, 28).

Activating point mutations leading to constitutive activation of the Ras proteins are associated with some 30% of all human malignancies (1), and these mutations render Ras insensitive to the regulatory action of GTPase activating proteins, leading to excessive and inappropriate signaling, resulting in the promotion of cellular transformation. However, while mutationally active ras genes are generally believed to act as dominant oncogenes, recent reports indicate that wild-type K- and N-Ras proteins have tumor suppressor activity (7, 32). Thus, K- and N-ras appear to exert a dual function in that they promote cancer development as gain-of-function oncogenes when mutated and inhibit cancer by tumor suppressor activity when wild type (proto-oncogene).

The high degree of homology between Ras proteins suggests that functional redundancy may exist among these proteins, yet mounting evidence exists for unique roles for the ras gene family members. For example, most human cancers with ras activating mutations are associated exclusively with a particular ras gene, suggesting tissue-specific activity: K-ras mutations are prevalent in lung (≥30%), colon (≥40%), and pancreatic (≥90%) cancers, while H-ras and N-ras activating mutations are associated with bladder cancer and myeloid leukemia, respectively (1). Furthermore, while N-ras and H-ras are dispensable for development both individually and in combination (10, 26), mice harboring a homozygous K-ras null mutation (K-ras<sup>−/−</sup>) are not viable and die between day 12.5 post coitum and term (depending on the genetic background) due to cardiac, liver, neurological, and hematopoietic defects (18, 19). Thus, of the classical ras gene family members (K-, N-, and H-ras), only K-ras is necessary for embryonic development. However, the findings that (i) N-ras<sup>−/−</sup> mice are healthy but N-ras<sup>−/−</sup>, K-ras<sup>−/−/−</sup> mice die in utero (18), (ii) K-ras<sup>−/−/−</sup> N-ras<sup>−/−</sup> mice exhibit a more severe phenotype than K-ras<sup>−/−/−</sup> mice (18), and (iii) fewer than expected H-ras<sup>−/−</sup> N-ras<sup>−/−</sup> mice survive embryogenesis (10) underline the essential role for K-Ras in development and imply partial functional overlap between different Ras proteins.

Understanding the role(s) of K-Ras in development and tissue function is an important route to gain insight as to how K-ras activating mutations promote neoplastic change. How-
ever, this is complicated by the fact that the \textit{K-ras} gene encodes two protein isoforms, K-Ras 4A and K-Ras 4B, of 189 and 188 residues, respectively, by alternative splicing of the fourth coding exons 4A and 4B, and \textit{K-ras} activations mutants that usually affect codons 12, 13, and 61 jointly affect both isoforms (5, 9, 21). The isoforms differ significantly at their C termini after residue 165, including the hypervariable domains and CAAX motifs. These regions are involved in membrane association, which is essential for Ras function (16) via a series of posttranslational modifications, which include isoprenylation, endoproteolysis, and methylation (25). However, these modifications differ between the isoforms due to the sequence differences at the hypervariable domain: K-Ras 4A, like N- and H-Ras, is palmitoylated at cysteine residues upstream of the CAAX motif, which are replaced with a polylysine domain in K-Ras 4B.

The difference in C-terminal modifications of Ras proteins leads to alternative trafficking pathways to the plasma membrane (3) and ultimately localization to different plasma membrane microdomains (23, 24). Indeed, reports that H-, N-, and K-Ras differentially affect Raf-1, phosphatidylinositol 3-kinase, and Rac (28, 29, 31) suggest that they elicit divergent biological responses by interacting with different subsets of downstream effectors. Thus, the posttranslational differences between K-Ras 4A and K-Ras 4B could affect their membrane localization and, therefore, interaction with different membrane targets. The specific association between K-Ras 4B and the guanine nucleotide exchange factor Smg GDS (27) and reports that the oncogenic mutant (G12V) K-Ras 4A and 4B differ in their ability to activate Raf-1, induce transformed foci, enable anchorage-independent growth, and promote cell migration in vitro (28) and that K-ras 4A and 4B are expressed differentially during mouse development and in adult tissues (22, 30) further suggest that they have distinct biological actions. Thus, the cooperative effects of both isoforms could account, at least in part, for the high frequency of \textit{K-ras} activating mutations in human cancers.

To examine the role of the individual \textit{K-ras} splice variants in development, gene targeting was used to delete exon 4A and generate \textit{K-ras} 4A-deficient mice. It was found that \textit{K-ras}m\textsuperscript{4A/4A} mice are healthy and fertile. The result demonstrates that expression of \textit{K-ras} 4A is dispensable for normal development, at least in the presence of functional K-Ras 4B.

**MATERIALS AND METHODS**

**Design of the \textit{K-ras} 4A targeting vector.** A nonisogenic targeting vector (pPTK/\textit{K}m\textsuperscript{4A}) was used to delete exon 4A of \textit{K-ras} to generate a mutant allele designated \textit{K-ras}\_m\textsuperscript{4A/4A}. The 2.8-kb 5’ and the 1.3-kb 3’ arms of homology were isolated by \textit{HindIII} and \textit{XbaI}-\textit{EcoRI} digests, respectively, of an 11.5-kb \textit{EcoRI} fragment (from BALB/c mice) stretching from upstream of exon 3 to upstream of exon 4B (13). The neomycin resistance cassette (containing a phosphoglycerate kinase 1 promoter) and the herpes simplex virus thymidine kinase gene cassette were employed for positive and negative selection, respectively (Fig. 1A).

**Production of \textit{K-ras}m\textsuperscript{4A/4A} embryonic stem cells.** The targeting vector was linearized with \textit{EcoRI}, and 150 μg of DNA was electroporated (800 V and 3 μF, Gene Pulser, Bio-Rad) into strain 129/Ola-derived HM-1 male mouse embryonic stem cells, which harbor an inactivating deletion in the X-linked \textit{Hprt} gene (20). Homologous recombination in colonies resistant to G418 (300 μg/ml; Invitrogen) and ganciclovir (2 μM; Sigma) was identified initially by PCR and confirmed by Southern analysis. Embryonic stem cell clones were screened with the primer set neo22 (5’-CGATAGAAGGGCAGTGCGTGCAGAAT-3’) and Ps4BA (5’-A TAACGTGACACTTGTCCTGGACT-3’), positioned in the neomycin cassette and exon 4B, respectively (Fig. 1A), which amplify a 2.1-kb product. The PCR conditions were 1.0 μM each primer (Sigma), 1× PCR buffer (Invitrogen), 1.5 mM MgCl\(_2\) (Invitrogen), 200 μM each deoxynucleoside triphosphate (Amersham), 1 unit of Taq DNA polymerase (Invitrogen), and 200 ng of genomic DNA per reaction. Following denaturing for 4 min at 94°C, DNA was amplified for 45 cycles (94°C for 1 min, 58°C for 1 min, and 72°C for 2.5 min).

Homologous recombination was confirmed by Southern blotting. Genomic DNA (20 μg) was digested with \textit{HindIII} or \textit{PvuII} and, following electrophoresis, was transferred to a nylon membrane (GeneScreen) in denaturing buffer (0.5 M NaOH, 1.5 M NaCl). The membrane was neutralized and prehybridized at 65°C for 2 h (10% dextran sulfate, 1% sodium dodecyl sulfate, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). 200 μg of herring sperm DNA per ml). The 3’ internal and 5’ external probes (Fig. 1A) were labeled with [\(^{32}\)P]dCTP by random priming (Stratagene) and hybridized to the membrane at 65°C overnight. Membranes were washed at room temperature with 2× SSC (twice for 5 min), at 65°C with 2× SSC-1% sodium dodecyl sulfate (twice for 30 min), then at room temperature with 0.1× SSC (twice for 30 min). Membranes were exposed to film overnight at −70°C.

**Production of \textit{K-ras} 4A-deficient mice.** Embryonic stem cells harboring the heterozygous deletion of exon 4A (\textit{K-ras}\_m\textsuperscript{4A/4A}) were injected into day 3.5 post coitum C57BL/6 blastocysts, which were implanted into the uteri of day 2.5 post coitum CD1 pseudopregnant recipients. Male chimeras were mated with 129/Ola or C57BL/6 females to generate inbred and crossed lines, and germ line transmission of embryonic stem cell markers was identified by coat color (light yellow and agouti, respectively).

Since long-range PCR proved unreliable for analyzing tail biopsy DNA, mice were genotyped with two different PCRs. In reaction 1, the primer set neo52 (5’-GATTGCGTGTGTCAGATATG-3’, neo22 (5’-CGATAGAA GGGCAGTGCGAGAATTG-3’), positioned in the neomycin cassette (Fig. 1A), amplify a 206-bp product in \textit{K-ras}\_m\textsuperscript{4A/4A} mice. In reaction 2, the primer set Ps4AS (5’-CATGTGAGAGATCCGCACTG-3’), and Ps4AA (5’-TACAGAGCAGGATCTTCTCTC-3’), positioned in exon 4A (Fig. 1A). A 72-bp product in wild-type and \textit{K-ras}\_m\textsuperscript{4A/4A} mice. In both reactions, DNA was denatured for 4 min at 94°C and amplified for 30 cycles at 94°C for 30 s and 58°C for 30 s. The genotypes were subsequently confirmed by Southern blotting with internal and external probes as described above.

In the inbred colony, first-generation \textit{K-ras}\_m\textsuperscript{4A/4A} males (which do not harbor the \textit{Hprt} null mutation) were mated with wild-type 129/Ola mice to generate an inbred stock that was wild type for \textit{Hprt}. In the crossbred colony, the \textit{Hprt} mutation was allowed to segregate. All animal work was carried out under Home Office license, with ethically approved methods as set out by the UK Coordinating Committee on Cancer Research in their guidelines on the Welfare of Animals in Experimental Neoplasia.

**RNA analysis.** K-ras 4A and 4B expression was determined in tissues from adult crossbred (129/Ola × C57BL/6) mice by reverse transcription-PCR (RT-PCR) with primers positioned within exons 1 and 4B, which amplify both isoforms in the same reaction (22). RNA was extracted with Trizol reagent (Invitrogen), and first-strand cDNA synthesis was performed with the SuperScript premplification system, with 1 to 5 μg of RNA (Invitrogen). For each sample, controls without reverse transcriptase were included and were negative in all cases.

**Histology.** Mice were sacrificed by cervical dislocation, and tissues were fixed overnight in 10% neutral buffered formalin except for the large and small intestines, which were fixed in Methacarn (methylene, chloroform, and glacial acetic acid, 4:2:1 by volume) overnight. Tissues were wax embedded, and 5-μm sections were cut and stained with hematoxylin and eosin.

**RESULTS**

**Generation of \textit{K-ras} 4A-deficient mice.** The targeting vector pPTK/\textit{K}m\textsuperscript{4A} was designed to replace exon 4A of \textit{K-ras} with a neomycin resistance cassette (Fig. 1A). Of 324 embryonic stem clones screened by PCR, 17 were positive for homologous recombination, amplifying the correct 2.1-kb PCR product (Fig. 1B). The genotype of seven of these clones was confirmed by Southern analysis with a 5’ external probe that identified a 5-kb wild-type and a 4-kb targeted band and a 3’ internal probe that identified a 2-kb wild-type and a 6-kb targeted band (Fig.
Of nine chimeras (generated from three different embryonic stem cell clones), five transmitted embryonic stem cell-derived coat color markers through the germ line. Since the effects of mutations, including the K-ras null mutation (18), can vary greatly with the genetic background, the consequence of deleting K-ras expression was examined in inbred (129/Ola) and crossbred (129/Ola/H11003/C57BL/6) strains of mice. The embryonic stem cell-derived offspring were genotyped at weaning by PCR (with the primer sets neo22/neo52 and Px4AS/Px4AA, which amplify the neomycin cassette and exon 4A, respectively; Fig. 2A) and by Southern blotting (with the 5′ external and 3′ internal probes; Fig. 2B), and it was found that K-ras<sup>tm4A−/−</sup> and wild-type mice were present in the expected Mendelian ratio (54 and 61, respectively; χ²₁ = 0.426, P = 0.514).

**K-ras 4A-deficient mice are healthy and fertile.** Offspring from crosses between K-ras<sup>tm4A−/−</sup> mice were genotyped by PCR (Fig. 2A) and Southern blotting (Fig. 2B). Wild-type, K-ras<sup>tm4A−/−</sup>, and K-ras<sup>tm4A−/tm4A−</sup> offspring were present in the expected Mendelian ratios on both crossbred (48, 101, and 58, respectively; χ²₁ = 1.087, P = 0.581) and inbred (11, 20, and 10, respectively; χ²₁ = 0.073, P = 0.964) genetic back-
K-ras 4A-deficient mice express the K-ras 4B splicing variant only. To confirm that the modification of the K-ras gene, by the introduction of the targeting vector, resulted in the deletion of the K-ras 4A splice variant, RT-PCR was used to examine K-ras expression in the large intestine of wild-type and K-ras<sup-tm4A/tm4A</sup> mice. Only the K-ras 4B splice variant was expressed by K-ras<sup-tm4A/tm4A</sup> mice (Fig. 3).

**K-ras 4A expression in wild-type adult mice.** To date, the expression of K-ras 4A has been examined in only a limited number of adult mouse tissues (22, 30). In the present study, the analysis was extended further to gain a comprehensive view of the overall pattern of K-ras 4A expression in wild-type adult mice. RT-PCR analysis found that K-ras 4A was expressed in the liver, large and small intestine, stomach, cecum, kidney, uterus, salivary gland, and seminal vesicles and only at low levels in the lung, pancreas, and bone marrow cells (Fig. 4). In ground results, indicating that all K-ras<sup-tm4A/tm4A</sup> mice develop normally. In addition, further breeding studies found that male and female K-ras<sup-tm4A/tm4A</sup> offspring were present in the expected Mendelian ratios in both crossbred (113 and 113, respectively) and inbred (20 and 25, respectively; \( \chi^2 = 0.556, P = 0.456 \)) stocks. Male and female K-ras<sup-tm4A/tm4A</sup> mice were fertile, and females successfully weaned their young.

Detailed histopathological analysis was undertaken of 24 crossbred mice at 3 months, including 12 wild-type and 12 K-ras<sup-tm4A/tm4A</sup> mice, with six males and six females in each cohort. No abnormalities were detected at necropsy, and examination of liver, kidney, large and small intestine, stomach, pancreas, spleen, thymus, heart, lung, brain, ovary, uterus, seminal vesicles, and testis found no difference between tissues from wild-type and K-ras<sup-tm4A/tm4A</sup> mice. Inbred K-ras<sup-tm4A/tm4A</sup> mice were also fertile and outwardly healthy, and histopathological analysis of a single animal at 3 months found no abnormalities. The oldest K-ras<sup-tm4A/tm4A</sup> mice in the crossbred colony are currently 8 months old, and these too are outwardly healthy.
contrast, K-ras 4B was expressed ubiquitously and was the only isoform expressed in brain, spleen, heart, adrenal gland, thymus, testis, and ovary. While both isoforms were expressed at similar levels in stomach and large intestine, K-ras 4A was the major isoform expressed in the cecum.

**DISCUSSION**

To gain further understanding concerning the role(s) of K-Ras in development and tissue function, we examined the pattern of K-ras 4A expression in wild-type adult mouse tissues and used gene targeting to delete K-ras 4A expression. While previous studies have established that expression of the K-ras gene is essential for mouse development (18, 19), here we found that, even though K-ras 4A is expressed during development and in adult tissues (22, 30; this study), all K-ras<sup>tmΔ4A/tmΔ4A</sup> mice on both inbred and crossbred genetic backgrounds developed normally, and the adult mice are fertile and healthy.

The finding that K-Ras 4A is dispensable for development suggests that the embryonic lethality of K-ras<sup>−/−</sup> mice (which do not express either isoform) may result solely from failure of expression of the K-Ras 4B isoform. However, the very fact that K-ras 4A is expressed during development, albeit in a spatially and temporally regulated manner (22), raises the possibility that the lethal K-ras<sup>−/−</sup> phenotype could result from loss of synergistic function of the two protein isoforms or else from loss of a critical function(s) that can be performed by either isoform, such that one or the other is necessary but in the absence of both development cannot occur. While the latter possibility is less likely, given that only the K-Ras 4B isoform is expressed ubiquitously throughout development (22), questions concerning whether expression of the K-Ras 4B isoform is essential for normal development and whether K-Ras 4A influences its action wait to be addressed by comparing the phenotypes of K-ras<sup>−/−</sup> and K-ras 4B-deficient mice on the same inbred genetic background. It is nevertheless important to stress that whatever the result of these future studies, our conclusion that K-Ras 4A is dispensable for normal mouse development in the presence of functional K-Ras 4B remains valid.

The C-terminal modifications of Ras proteins are essential for protein function (16), and functional differences between Ras proteins may reflect, at least in part, these different modifications. Therefore, the findings by Hancock et al. that N-, H-, and K-Ras 4A undergo similar C-terminal modifications which involve palmitoylation of cysteine residues upstream of the common CAAX motif (14) are significant because these proteins are dispensable for normal development. Furthermore, the viability of K-ras<sup>tmΔ4A/tmΔ4A</sup> mice is unlikely to reflect a compensatory upregulation of these closely related Ras proteins, since the simultaneous deletion of both K-ras splice variants does not result in the upregulation of H-ras or N-ras expression in either tissues or fibroblast cultures from K-ras<sup>−/−</sup> embryos (18). Likewise, H-ras<sup>−/−</sup> N-ras<sup>−/−</sup> mutant mice show no change in K-ras expression (10).

Importantly, since N-ras<sup>−/−</sup>, H-ras<sup>−/−</sup>, and K-ras<sup>tmΔ4A/tmΔ4A</sup> mice are fertile, it is now possible, by crossing these mice, to formally test whether the viability of K-ras<sup>tmΔ4A/tmΔ4A</sup> mice is indeed independent of N- and H-ras expression. Also, since K-Ras 4B, unlike K-Ras 4A, associates with the plasma mem-
why they are linked with particular types of epithelial cell-derived tumors.

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REFERENCES