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The Dual-Specificity Protein Phosphatase DUSP9/MKP-4 Is Essential for Placental Function but Is Not Required for Normal Embryonic Development†

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To elucidate the physiological role(s) of DUSP9 (dual-specificity phosphatase 9), also known as MKP-4 (mitogen-activated protein kinase [MAPK] phosphatase 4), the gene was deleted in mice. Crossing male chimeras with wild-type females resulted in heterozygous ($DUSP9^{+/-}$) females. However, when these animals were crossed with wild-type ($DUSP9^{+/y}$) males none of the progeny carried the targeted DUSP9 allele, indicating that both female heterozygous and male null ($DUSP9^{-/y}$) animals die in utero. The DUSP9 gene is on the X chromosome, and this pattern of embryonic lethality is consistent with the selective inactivation of the paternal X chromosome in the extraembryonic tissues of the mouse, suggesting that DUSP9/MKP4 performs an essential function during placental development. Examination of embryos between 8 and 10.5 days postcoitum confirmed that lethality was due to a failure of labyrinth development, and this correlates exactly with the normal expression pattern of DUSP9/MKP-4 in the trophoblast giant cells and labyrinth of the placenta. Finally, when the placental defect was rescued, male null ($DUSP9^{-/y}$) embryos developed to term, appeared normal, and were fertile. Our results indicate that DUSP9/MKP-4 is essential for placental organogenesis but is otherwise dispensable for mammalian embryonic development and highlights the critical role of dual-specificity MAPK phosphatases in the regulation of developmental outcomes in vertebrates.

Dual-specificity (Thr/Tyr) protein phosphatases play an important role in the dephosphorylation and inactivation of mitogen-activated protein kinases (MAPKs) in eukaryotic cells (2, 19, 32). These MAPK phosphatases (MKPs) act in direct opposition to the dual-specificity MAPK kinases to regulate the magnitude and duration of MAPK activation and hence the physiological outcome of signaling. To date, 10 distinct dual-specificity MKPs have been isolated and characterized in mammalian cells (32). Based on sequence homology, substrate selectivity, and subcellular localization, these enzymes can be divided into three distinct subfamilies (2, 19, 32). The first comprises DUSP1/MKP-1/CL100, DUSP2/PAC-1, DUSP4/MKP-2, and DUSP5/hVH-3, all of which are inducible nuclear MKPs. The second group comprises DUSP8/hVH-5, DUSP10/MKP-5, and DUSP16/MKP-7, which preferentially dephosphorylate the stress-activated MAPKs such as c-Jun amino-terminal kinase (JNK) and p38. The final group comprises a subfamily of three genes, DUSP6/MKP-3/Pyst1, DUSP7/MKP-X/Pyst2, and DUSP9/MKP-4/Pyst3, encoding cytoplasmic MKPs that preferentially recognize and inactivate classical ERK1 and -2 MAPKs in mammalian cells.

In recent years, much progress has been made in understanding the regulation, catalytic mechanism, and substrate selectivity of the MKPs. The selective dephosphorylation of ERK1/2 by DUSP6/MKP-3 is accompanied by the formation of a stable complex between these two enzymes in which MAPK recognition and binding are mediated by a conserved motif within the amino-terminal noncatalytic domain of the phosphatase (13, 22). Furthermore, MAPK binding is accompanied by catalytic activation of DUSP6/MKP-3, as revealed by a greatly increased ability to hydrolyze the chromogenic substrate *para*-nitrophenylphosphate in vitro (3, 10, 29). The observation that other MKPs, including DUSP7/MKP-X/Pyst2, DUSP9/MKP-4/Pyst3, DUSP1/MKP-1, and DUSP4/MKP-2, also undergo catalytic activation on binding to their substrate MAPKs suggests that this is a general mechanism regulating substrate selectivity for the MKPs (3, 4, 6, 8, 27).

Despite a wealth of data obtained in cultured cells supporting a role for these enzymes in regulating MAPK signaling, until recently there was little direct evidence that these enzymes play important physiological roles in mammals. Deletion of the murine gene encoding DUSP1/MKP-1 results in apparently normal and fertile animals (7). More recently, the murine gene encoding DUSP10/MKP-5, a phosphatase which targets the stress-activated MAPKs JNK and p38 in cultured cells, was deleted (35). These animals have defects in both innate and adaptive immune responses resulting from a failure to regulate JNK signaling, and this model provides the first demonstration of a nonredundant role for this class of enzymes in regulating MAPK signaling.

In the present study, we have focused on the subfamily of MKPs which preferentially recognize and inactivate classical

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ERK1 and -2 MAPKs in mammalian cells. We recently showed that the murine DUSP6/MKP-3 gene is expressed at sites of fibroblast growth factor (FGF) signaling during embryonic development, indicating that it may play a role in regulating MAPK activation downstream of the FGF receptor (5). This has been confirmed in chicken embryos, where DUSP6/MKP-3 is responsible for negative feedback regulation of FGF-induced ERK activation in developing limbs, neural plate, and somites (9, 18, 28). In contrast, DUSP9/MKP-4/Pyst3 is highly expressed in the placenta and developing liver but at lower levels elsewhere during early development and organogenesis (6).

To investigate the physiological role of DUSP9/MKP-4, we have generated knockout mice by gene targeting in embryonic stem (ES) cells. Here we report that loss of DUSP9/MKP-4 function results in embryonic lethality due to placental insufficiency and, in particular, a failure of normal labyrinth development. However, despite the fact that DUSP9/MKP-4 is expressed at several sites in developing embryos, including the liver, when the placental defect is rescued male null ($DUSP9^{-/y}$) embryos develop to term, appear normal, and are fertile. This is the first demonstration of an essential function for a member of this subfamily of ERK-specific MKPs in mammals and emphasizes the critical role played by these enzymes in regulating the developmental outcome of MAPK-mediated signaling events in vertebrates.

MATERIALS AND METHODS

Construction of the targeting vector and transfection of ES cells. The isolation and characterization of the murine DUSP9/MKP-4 gene have been described previously (6). A targeting vector was constructed by using the en2A-IRESβgeoPA cassette to replace exons 2 and 3 of DUSP9. This cassette contains a selectable marker (neomycin) and a gene encoding β-galactosidase under the control of a picornavirus IRES element (20). This strategy for selecting ES cells where homologous recombination has occurred depends on the expression of DUSP9 in ES cells, which was confirmed by Northern blotting (not shown). A 2.4-kb EcoRI-PmeI genomic fragment constituted the 5′ region of homology and a 2.7-kb BstXI-SpeI fragment the 3′ region of homology. The 1.9-kb region between the PmeI site in intron 1 and the BstXI site in the 3′ untranslated region was replaced with the IRES-βgeo cassette from plasmid pGT1.8 IRES-βgeo (provided by Austin Smith, Institute for Stem Cell Research, University of Edinburgh). The targeting construct was linearized with SpeI, purified, and transfected into GK 129/1 ES cells from 129 (P2) Ola mice by electroporation, and colonies were selected for G418 resistance.

Screening strategy and genotyping. Genomic DNA was isolated from G418-resistant ES cell clones, digested with XbaI and HindIII, transferred to nylon membrane (Hybond N+; Amersham), and analyzed by Southern blotting using a 700-bp XbaI-EcoRI DUSP9 genomic DNA fragment as a probe. This fragment lies immediately upstream of the 5′ region of homology and hybridizes to a 3.5-kb fragment in the wild-type DUSP9 gene and a 5-kb fragment in correctly targeted clones due to the presence of a HindIII site in the IRES-βgeo cassette. Positive clones were expanded and examined for correct homologous recombination at the 3′ end by Southern blotting of genomic DNA digested with NcoI using a 677-bp fragment of the IRES-βgeo cassette neomycin resistance gene as a probe. This probe hybridizes to a 3.8-kb NcoI fragment which is present only when the IRES-βgeo cassette is correctly inserted relative to an NcoI site which lies just outside the region of 3′ homology between the targeting construct and wild-type allele. Two correctly targeted clones were injected into blastocysts from C57BL/6J mice and transferred into pseudopregnant mice. Chimeric offspring which showed a high degree of ES cell contribution, as judged by coat color, were crossed with C57BL/6J mice, and offspring were genotyped by analysis of either tail tip or yolk sac DNA using multiplex PCR. A common antisense primer (3164R; 5′-ACCCTATCTCCTCCGGTGGCAGTTTGACCACC-3′) was used in combination with a DUSP9-specific sense primer (2841F; 5′-ACCTGTGGG AGCAGACCTGGCCACTTTGACTCG-3′) and a sense primer derived from the 3′ end of the IRES-βgeo cassette (6466F; 5′-TGATCCTCCAGCGCGGG GATCTCATGCTGG-3′). PCR conditions were 94°C for 2 min and then 30 cycles of 94°C for 30 s, 63.9°C for 1 min, and 72°C for 2 min, followed by a final extension step of 72°C for 7 min. Products were analyzed using 2% agarose gel electrophoresis. All experiments were carried out in accordance with the 1986 Animal Scientific Procedures Act and after local ethical review. All mice were maintained under standard animal house conditions with ad libitum access to standard rodent diet and water.

Tetraploid aggregations. Tetraploid embryos were generated by electrofusion of two-cell embryos (F_2 embryos from a C57BL/6J × CBA cross). Fused embryos were cultured overnight in KSOM medium (23). Following removal of the zona pellucida by acid Tyrodes treatment, aggregation drops were established which contained two tetraploid embryos to each 2.5-day postcoitum (dpc) embryo collected from the DUSP9 female heterozygote × wild-type matings. Following a further overnight culture, aggregated embryos were transferred to the uteri of E2.5 pseudopregnant B6CBAF1 foster females and offspring were genotyped by analysis of tail tip DNA using multiplex PCR as before. As controls, DUSP9 (heterozygote × wild-type) embryos were transferred without aggregation to tetraploid embryos and in addition transfers were performed from aggregations of tetraploid embryos alone.

Histology, immunohistochemistry, and *lacZ* staining. Entire conceptuses and placentas were isolated from staged pregnancies and fixed overnight in 4% paraformaldehyde. Following dehydration in methanol, tissues were embedded in paraffin and sectioned (4 μm). Tissue sections were either stained with hematoxylin and eosin prior to histological examination or prepared for immunohistochemistry analysis. DUSP9 was detected using a sheep polyclonal antiserum (6); ERK1/2, phospho-ERK2, p38, and phospho-p38 were detected using antibodies from Cell Signaling Technologies according to the manufacturer's instructions. β-Galactosidase staining of whole embryos or tissues was performed exactly as described by Henkemeyer et al. (16).

Western blotting. Cultured ES cells, 10.5-dpc placentas, and adult kidneys or testes were lysed in a buffer containing 20 mM Tris acetate (pH 7.0), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 5 mM $NaPP_i$, 50 mM NaF, 1 mM sodium orthovanadate, 0.27 M sucrose, and 10 mM β-glycerophosphate supplemented with Complete EDTA-free protease inhibitor cocktail tablets (Roche). Following brief centrifugation, supernatant containing 7.5 to 10 μg soluble protein was analyzed using the NuPAGE electrophoresis system (4 to 12%; Invitrogen) and transferred to a 0.45-μm polyvinylidene difluoride membrane (Millipore). Immunoblotting was then performed using the following antibodies: sheep polyclonal DUSP9 antiserum (6), tubulin β Ab-3 (Neomarkers), ERK1/2 and phospho-ERK2, ERK5, p38 and phospho-p38, JNK and phospho-JNK, and Akt and phospho-Akt (all from Cell Signaling Technologies).

RESULTS

Deletion of DUSP9/MKP-4. Homologous recombination was used to delete the DUSP9 gene in murine ES cells. We designed a targeting construct in which an internal 1.9-kb PmeI-to-BstXI MKP-4 genomic fragment was replaced with an IRES-βgeo cassette (Fig. 1A). The deleted region spans exons 2 and 3, which encode amino acids 194 to 452 of DUSP9/MKP-4 and includes the entire catalytic domain of the protein, including the protein tyrosine phosphatase active-site motif HCXXGXXRS (6). The linearized targeting vector was electroporated into GK 129/1 ES cells, and Southern blot analysis of genomic DNA prepared from 472 independent G418-resistant ES cell clones identified two clones in which the desired homologous recombination event had occurred (Fig. 1B and C). As GK 129/1 ES cells are male derived and the DUSP9 gene is located on the X chromosome (6), a wild-type DUSP9 allele is absent in these cells (Fig. 1B) and the DUSP9/MKP-4 protein, which is readily detected by Western blotting of wild-type ES cell lysates, is not expressed (Fig. 1D). Interestingly, we see no evidence of abnormal phosphorylation of either ERK1/2 or p38 MAPKs in null versus wild-type ES cell lysates, indicating that loss of DUSP9/MKP-4 does not lead to any increase in the basal activity of these MAPKs (Fig. 1D). In addition, both targeted clones grow and divide at the same rate

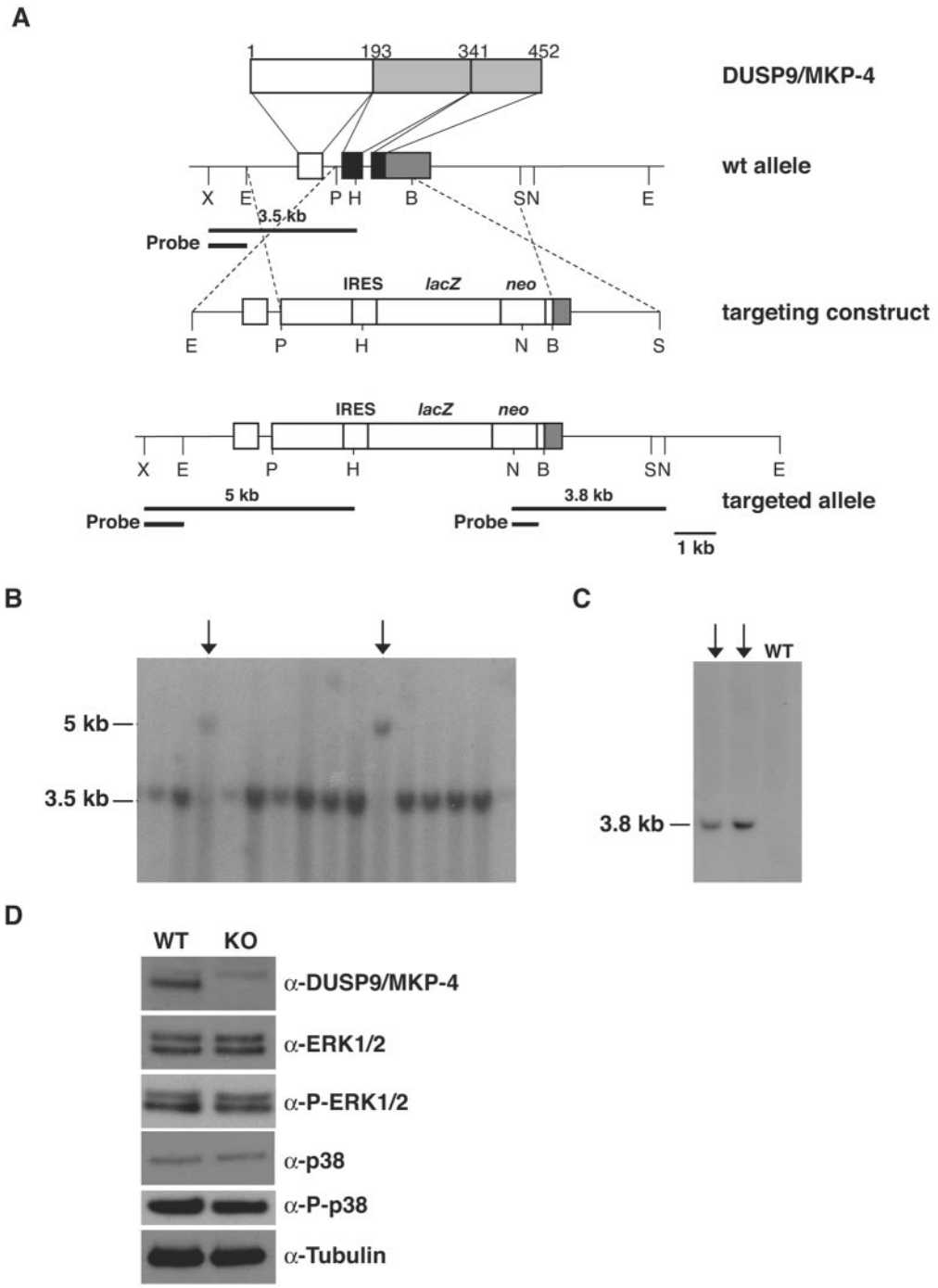

FIG. 1. Generation of mouse ES cells lacking DUSP9/MKP-4 by homologous recombination. (A) Schematic showing the domain structure of the DUSP9/MKP-4 protein, the wild-type (wt) DUSP9 allele, the targeting construct, and the targeted allele. Deleted regions of the protein are numbered by residue and shaded light. The coding regions of exons 2 and 3 are shown by black boxes, while the 3′ untranslated sequence is shaded dark. Recombination events are indicated by dashed lines and show the replacement of a 1.9-kb PmeI-to-BstXI DUSP9/MKP-4 genomic fragment with the IRES-βgeo cassette. Restriction enzymes are indicated as follows: B, BstXI; E, EcoRI; H, HindIII; N, NcoI, P, PmeI; S, SpeI; X, XbaI. (B) An example of 5′ Southern blot analysis of ES cell genomic DNA from 15 G418-resistant clones following digestion with XbaI and HindIII using the external XbaI-EcoRI probe as indicated in panel A. The migration of the 5-kb (targeted) and 3.5-kb (wild-type) hybridizing fragments is shown on the left, and the two positive clones are arrowed. (C) Southern blot analysis of the 3′ end of the targeted allele in these clones following digestion with NcoI using a fragment of the *neo* resistance gene as a probe. Correct targeting is verified by the presence of a 3.8-kb hybridizing band corresponding to the NcoI restriction fragment shown in panel A. (D) Western blot analysis of protein lysates from wild-type (WT) or null (KO) ES cells. The protein species detected are indicated on the right. Tubulin is included (bottom) as a loading control.

TABLE 1. Genotype analysis of progeny from DUSP9 female heterozygote (+/−) × DUSP9 wild-type male (+/y) crosses

Age (no. of mice)	% of mice		
	Wild type	Knockout	Heterozygous
8.5 dpc (37)	46	38	16
9.5 dpc (49)	45	18	37
10.5 dpc (121)	50	28	21
11.5 dpc (11)	100	0	0
14.5 dpc (10)	100	0	0
Postnatal (72)	100	0	0

as wild-type ES cells (data not shown), indicating that DUSP9/MKP-4 function is not essential for ES cell viability or growth.

Deletion of DUSP9/MKP-4 causes embryonic lethality. Chimeric mice were generated by injecting null ES cells into C57BL/6 blastocysts with both clones, resulting in germ line transmission of the disrupted DUSP9 allele. The resulting heterozygous female mice were of normal size and fertile. However, when these mice were crossed with wild-type males none of the progeny analyzed (72 animals) at 3 weeks after birth carried the targeted DUSP9 allele (Table 1), indicating that in this second cross both female heterozygous and male null animals die in utero. This pattern of embryonic lethality among the female heterozygotes is consistent with the selective inactivation of the paternal X chromosome in the extraembryonic tissues of the mouse (30) and suggests that DUSP9/MKP-4 performs an essential function during placental development.

To investigate this further, we genotyped the embryos from litters at 8.5 dpc and found normal Mendelian ratios of wild-type, heterozygous, and null DUSP9 embryos (Fig. 2A and Table 1). Staged matings revealed that both heterozygous and null animals persisted through 9.5 to 10.5 dpc, but we could find no heterozygous or null embryos in litters analyzed at 11.5 and 14.5 dpc (Table 1). Furthermore, analysis of litters at 10 dpc revealed that while 90% (27 of 30) of wild-type embryos had a detectable heartbeat, this was seen in only 4.5% (1 of 22) of DUSP9 null animals, suggesting that loss of DUSP9/MKP-4 function results in lethality at around 10.5 dpc (not shown). Examination of embryos at 8.5 dpc revealed no significant differences in morphology among wild-type, heterozygous, and null embryos. However, comparison of embryos from litters at 10.5 dpc using typical morphological criteria revealed that both heterozygous and null animals were retarded in growth by approximately 1 day compared to their wild-type littermates, and many exhibited pericardial effusion (Fig. 2B). Both growth retardation and cardiovascular abnormalities have been observed to result from gene knockouts which affect placental development and function. These include loss of MAPK signaling cascade components such as p38α, ERK5, MEK1, and MEKK3 (1, 12, 25, 34).

DUSP9/MKP-4 is essential for placental development. We next examined placentas from wild-type, heterozygous, and null embryos. Firstly, as predicted by genetics, DUSP9/MKP-4 protein is completely absent from both heterozygous and null placentas when analyzed by Western blotting of tissue lysates (Fig. 3B). In addition, histological comparison of stained tissue sections revealed gross abnormalities in DUSP9/MKP-4 null placentas at 10.5 dpc. While the maternal decidua appeared completely normal, the labyrinth layer was much thinner and more compact compared to sections from wild-type placenta (Fig. 2C). Furthermore, enucleate maternal erythrocytes were restricted to the maternal side of the labyrinth, while nucleated fetal erythrocytes remained in the vessels of the allantois and chorion (Fig. 2C). Identical results were obtained when heterozygous placentas were examined (not shown). The inability to form a functional labyrinth probably results from either slowing or arrest of villus formation and the subsequent growth of blood vessels into these structures (26). Examination of wild-type and null placentas at day 9.5 again shows morphological differences with an apparent underdevelopment of the spongiotrophoblast layer which lies immediately beneath the trophoblast giant cells (supplemental Fig. S1). These defects correlate exactly with the wild-type expression pattern for DUSP9/MKP-4 protein. Immunohistochemical detection reveals expression in the trophoblast giant cells, spongiotrophoblast layer, and labyrinth of the normal placenta, which is absent in sections from null placentas (Fig. 3A).

Abnormal phosphorylation of MAPKs is not detected in placental tissues lacking DUSP9/MKP-4. Yeast two-hybrid assays reveal that among a panel of nine mammalian MAPKs, ERK1 and -2 are the strongest binding partners for DUSP9/MKP-4, with significant interaction also detected between DUSP9/MKP-4 and p38α (6). This binding affinity is reflected in the ability of recombinant ERK2 to stimulate the catalytic activity of DUSP9/MKP-4 in vitro (3, 6). However, when expressed in mammalian cells, DUSP9/MKP-4 is able to dephosphorylate and inactivate both ERK2 and p38α (6). Overall, these results suggest that DUSP9/MKP-4 targets ERK1 and -2 and possibly also p38a in vivo and this prompted us to examine the phosphorylation and activity of MAPK signaling pathways in cells and tissues lacking MKP-4.

Firstly, we examined the phosphorylation state of ERK1/2, p38, and JNK MAPKs in placental lysates from wild-type, knockout, and heterozygous embryos at day 10.5 (Fig. 3B). In addition, the phosphorylation state of the ERK5 MAPK was monitored by Western blotting, where a marked phospho shift would indicate activation (Fig. 3B). No consistent differences were seen in either the levels or phosphorylation state of any of these MAPKs, nor was any change noted in the levels or phosphorylation of Akt, a substrate of the growth factor-activated phosphatidylinositol 3-kinase pathway. In agreement with these results, immunocomplex kinase assays also failed to reveal any significant differences in the activities of ERK2 and p38 MAPKs and analysis of placental lysates from embryos at day 8.5 gave identical results (data not shown). Given that MAPKs are expressed in both the maternal and fetal components of the developing placenta (24), it is possible that any changes in these activities occurring only in the fetal component might be underestimated by analysis of total tissue lysates. To address this problem, we examined the phosphorylation state of both ERK1/2 and p38 by immunohistochemical staining of tissue sections from wild-type and knockout placentas using phospho-specific antibodies against ERK1/2 and p38 MAPKs. Phospho-ERK and phospho-p38 are readily detected in tissues that also express DUSP9/MKP-4, particularly in the trophoblast giant cells and spongiotrophoblast layer. However, no significant differences in the intensity of staining were de-

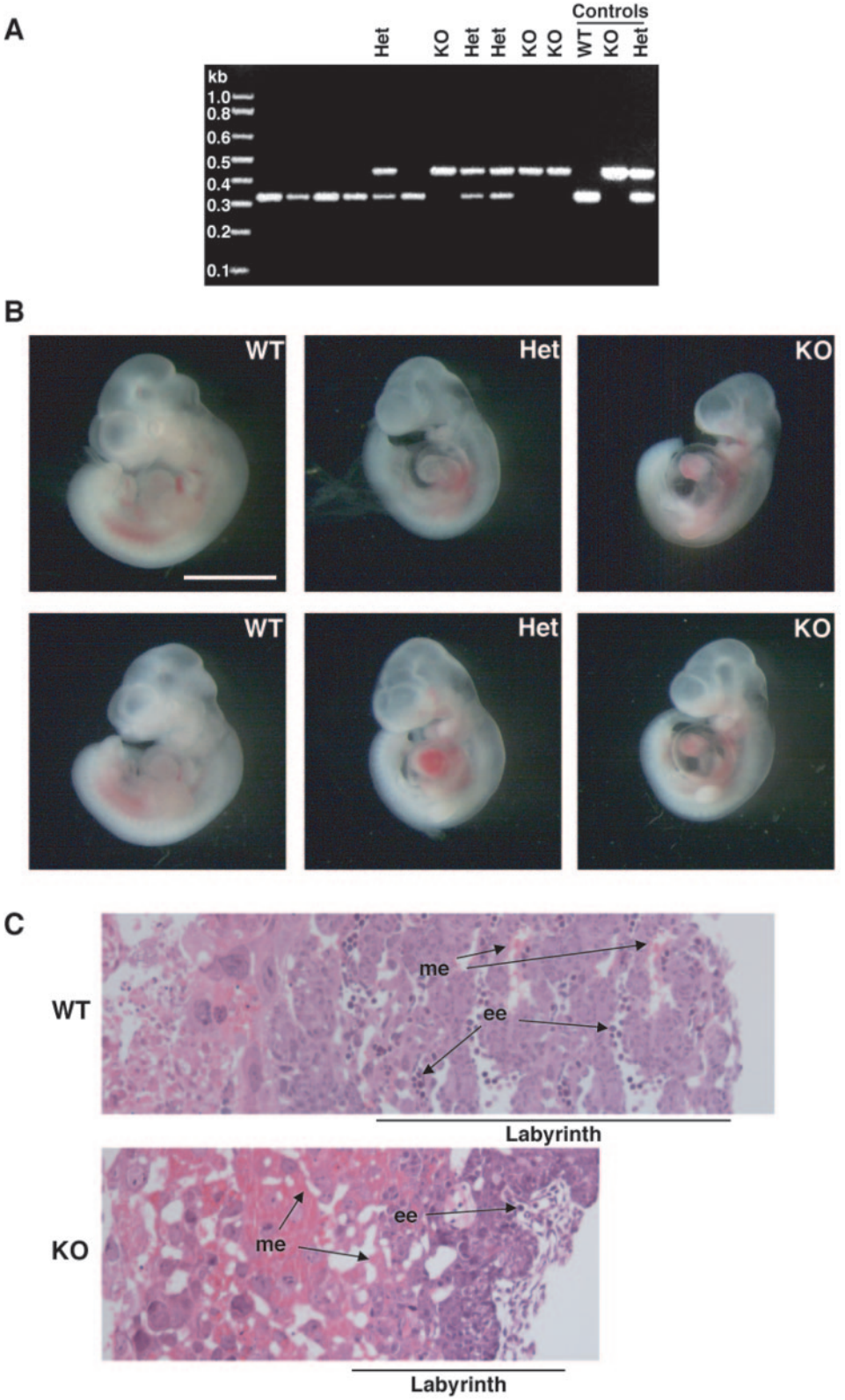

FIG. 2. Loss of DUSP9/MKP-4 causes placental defects that result in embryonic lethality. (A) Multiplex PCR assay of genomic DNA from a litter of 8.5-dpc mouse embryos resulting from a DUSP9 heterozygote × wild-type (WT) cross. Shown are the numbers of WT, heterozygous (Het), and null (KO) embryos. The migration of the KO and WT PCR products is shown on the right. DNA size markers are shown on the left with Het and KO embryos identified above. Control PCRs for WT, KO, and Het DNAs are shown on the right. (B) Phenotype of freshly dissected embryos at 10.5 dpc. Note the growth retardation exhibited by both the Het and KO embryos compared with their WT littermates. The scale bar represents 2 mm (top left). (C) Representative hematoxylin-and-eosin-stained sections of WT and KO 10.5-dpc placentas. Note the dramatically reduced thickness of the labyrinth layer of the DUSP9/MKP-4 KO placenta and the relative absence of blood vessels. In the WT placenta, the maternal erythrocytes (me) and embryonic erythrocytes (ee) are well distributed through the vessels of the labyrinthine layer. In contrast, in the KO placenta maternal erythrocytes remain on the maternal side of the labyrinth while the embryonic erythrocytes are found in the vessels of the allantois and chorion.

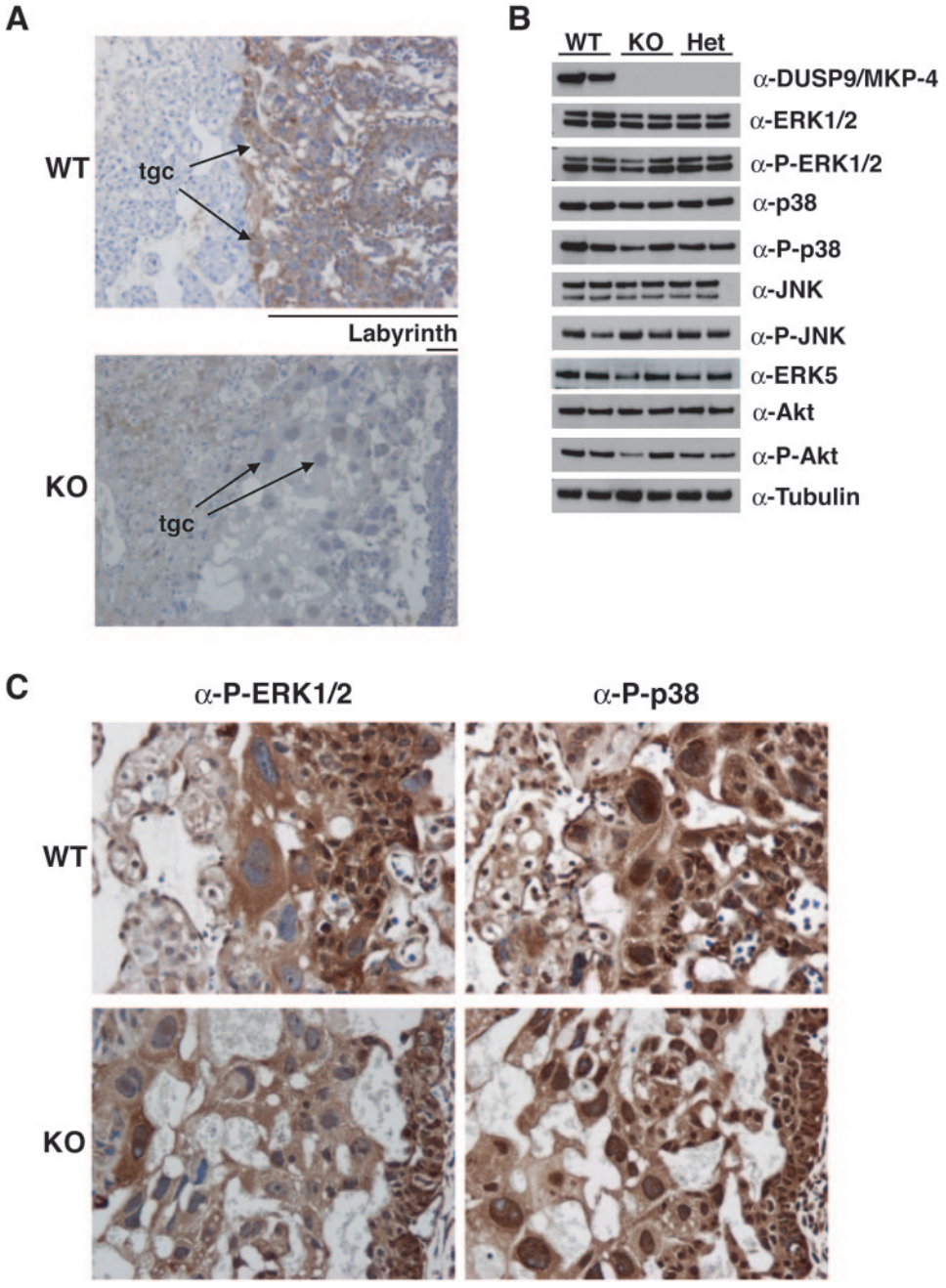

FIG. 3. Loss of DUSP9/MKP-4 does not lead to significant changes in the basal phosphorylation state of MAPKs in placental tissues. (A) Representative sections from wild-type (WT) and null (KO) 10.5-dpc placentas stained using a polyclonal antiserum against DUSP9/MKP-4. The labyrinth layer is indicated by a bold line, and trophoblast giant cells (tgc) are arrowed. (B) Western blot analysis of protein lysates from two independent WT, KO, and heterozygous (Het) 10.5-dpc placentas. The protein species detected are indicated on the right, and tubulin is included (bottom) as a loading control. (C) Representative sections from WT and KO 10.5-dpc placentas stained using antibodies against the phosphorylated (active) forms of ERK1/2 (left) or p38 MAPK (right).

tected when comparing wild-type and knockout tissue sections (Fig. 3C).

DUSP9/MKP-4 is not essential for embryonic development. The fusion of diploid mutant and tetraploid wild-type embryos can circumvent the embryonic lethality caused by defects in placental development (14). Tetraploid "rescue" can thus be used to verify that the primary defect lies in the trophoblast lineage and also allows the dissection of any primary defects within the embryo itself from the secondary consequences of placental insufficiency. When tetraploid wild-type and diploid embryos collected from DUSP9/MKP-4 female heterozygote × wild-type matings were aggregated, animals developed to term. Genotyping of four litters revealed that among 19 animals born, there were two male DUSP9/MKP-4 null mice and

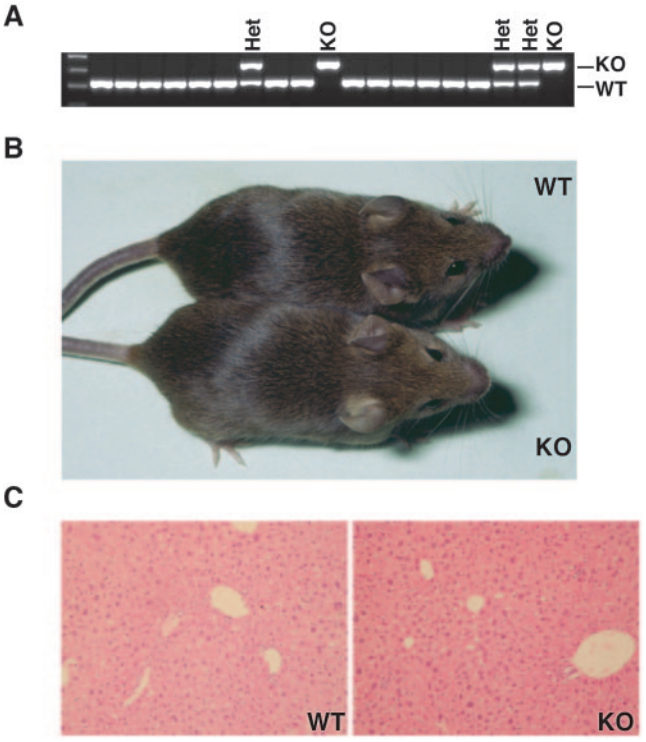

FIG. 4. DUSP9/MKP-4 is not essential for normal embryonic development. (A) Multiplex PCR assay of tail tip genomic DNA from a litter of mice following tetraploid aggregation. Shown are the numbers of wild-type (WT), heterozygous (Het), and null (KO) embryos. The migration of the KO and WT PCR products is shown on the right, and Het and KO embryos are identified above. DNA size markers are shown on the left. (B) Postweaning young adult male KO mouse photographed with a male WT littermate. (C) Representative hematoxylin-and-eosin-stained sections of livers from either WT (left) or KO (right) males showing normal tissue morphology in the MKP-4 null animals.

three female heterozygotes (Fig. 4A). The null mice were indistinguishable from their male wild-type littermates, were of normal weight, and displayed no apparent behavioral abnormalities (Fig. 4B).

A major site of DUSP9/MKP-4 mRNA expression during mouse embryogenesis is the developing liver (6). However, in adult mouse and human tissues, DUSP9 transcripts are not detected in this organ and expression is restricted to the kidney, testis, and white adipose tissues (6, 21, 33). The insertion of a β-galactosidase cassette into the DUSP9 locus allowed us to study the expression of the gene during embryonic development in female heterozygous ($DUSP9^{+/-}$) animals and expression of the *lacZ* reporter confirmed previous results obtained by whole-mount in situ hybridization (6). In particular, robust β-galactosidase staining is seen in the liver bud at 9 dpc and is maintained through 9.5 and 11 dpc to formation of the definitive liver (Fig. S2 in the supplemental material). However, by 12.5 dpc expression has declined noticeably and is totally absent by 13.5 dpc and beyond (not shown). In order to study the possible impact of DUSP9/MKP-4 loss on organ development and function, two male null animals and two male wild-type littermates were sacrificed and tissues examined.

We initially compared the livers of wild-type and DUSP9/MKP-4 null animals. These organs were identical in size and external appearance. Furthermore, stained liver sections from DUSP9/MKP-4 null animals were of normal morphology (Fig. 4C) and as seen previously (6), DUSP9/MKP-4 protein was undetectable in the livers of both wild-type and null animals (data not shown). This, coupled with the normal phenotype of our null animals, indicates that despite the robust expression observed in mouse embryos, DUSP9/MKP-4 is dispensable for normal liver development and function. We next examined two adult tissues in which DUSP9 mRNA expression can be detected by Northern blotting, namely, the kidney and testis.

β-Galactosidase staining of sectioned kidneys from female DUSP9/MKP-$4^{+/-}$ animals revealed expression in both the cortex and medulla (Fig. S2 in the supplemental material). We next compared organs from DUSP9/MKP-$4^{+/+}$ and DUSP9/MKP-$4^{-/y}$ animals. DUSP9/MKP-4 null kidneys were of normal size and appearance, and stained tissue sections were of normal morphology. Immunostaining revealed that MKP-4 expression was limited to the thick ascending limb of the loop of Henle (Fig. 5), which is consistent with expression in both superficial (cortical) and juxtamedullary nephrons. Testes from DUSP9/MKP-4 null males were of normal morphology, and immunostaining of wild-type and null tissue sections revealed that DUSP9/MKP-4 expression was confined to the seminiferous tubules, with little or no expression in interstitial cells (Fig. 5). In order to monitor the activation state of ERK2 and p38 MAPKs in these organs, we performed both Western blotting of whole-tissue lysates and also immunohistochemical staining of tissue sections from wild-type and knockout animals using phospho-specific antibodies against ERK1/2 and p38 MAPKs (Fig. 6). Phospho-ERK and phospho-p38 are readily detected in both kidney and testis tissues. However, no significant differences in the levels of either unphosphorylated or phosphorylated MAPKs were detected. Finally, we wished to know if the physiological function of either organ system was compromised in our MKP-4 null animals. The small number of null animals available precluded assessment of renal function. However, gross testicular function was addressed by assessing the fertility of our DUSP9/MKP-4 null males prior to sacrifice. Both male nulls were paired at the same time as two wild-type male littermates and mated successfully to produce litters containing the expected Mendelian ratio of wild-type males and female heterozygote animals.

DISCUSSION

Deletion of DUSP9/MKP-4 causes a pattern of embryonic lethality in both female heterozygous and male null animals, which indicates an essential function in placental development. This was confirmed by analysis of extraembryonic tissues from these animals and by performing tetraploid aggregations to "rescue" both heterozygous and null embryos. The developmental defect is characterized by a failure to form a functional labyrinth, and this is entirely consistent with the expression pattern of the DUSP9/MKP-4 protein, which is found in the trophoblast giant cells, spongiotrophoblast, and labyrinth of the wild-type placenta. Our previous studies have identified ERK1/2 and p38 MAPKs as potential targets for DUSP9/MKP-4 in vitro and in vivo (6). Despite this and the clear defects in placental tissues lacking DUSP9/MKP-4, we can find

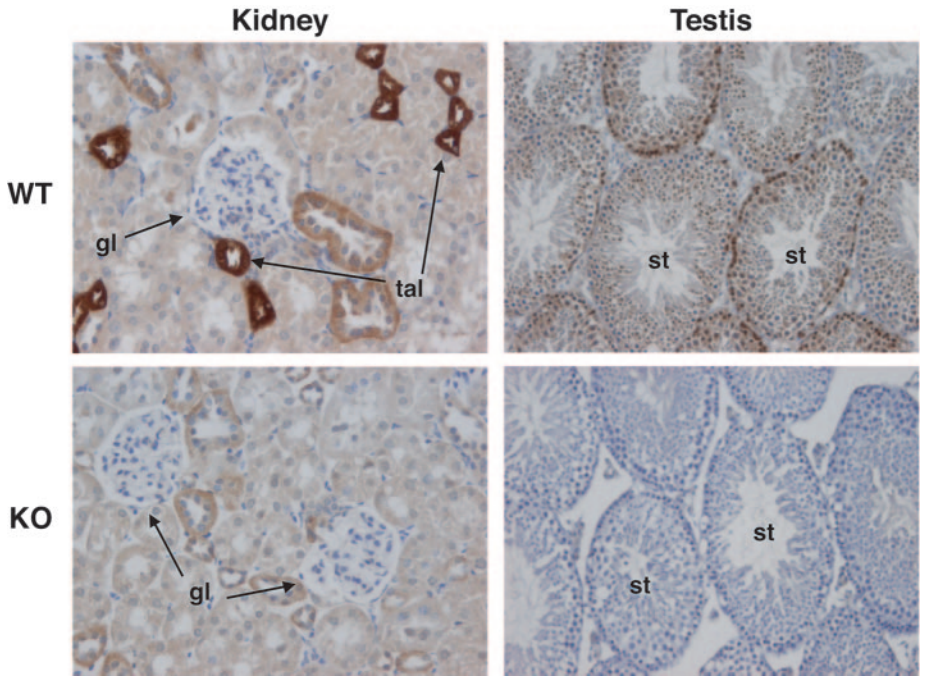

FIG. 5. Representative sections of kidney (left panels) and testis (right panels) tissues from either wild-type (WT) or null (KO) males stained with a polyclonal antibody against DUSP9/MKP-4. The thick ascending limb (tal) of the loop of Henle and the glomeruli (gl) are indicated by arrows in the kidney sections, while the seminiferous tubules (st) are indicated in the testis tissue.

no evidence of gross abnormalities in either the levels or activities of these MAPKs using a number of approaches, including immunocomplex kinase assays and Western blotting or immunohistochemical analysis using phospho-specific antibodies against ERK1/2 and p38.

There are a number of reasons which may account for our inability to correlate DUSP9/MKP-4 loss with altered regulation of MAPK signaling. Firstly, in the absence of DUSP9/MKP-4, abnormally elevated levels of MAPK activity may persist only for a very limited time during placental development before other compensatory mechanisms act to down-regulate signaling. However, this transient increase over the normal level of activity may be sufficient to preclude normal labyrinth development. Secondly, we do not know the upper and lower tolerances for MAPK activities which are permissive for normal developmental processes and it is possible that the alteration in MAPK regulation caused by DUSP9/MKP-4 loss is below a level which we can discriminate using the methods of tissue analysis employed here. Our analysis of the impact of DUSP9/MKP-4 deletion on MAPK signaling would be greatly aided by the isolation of cell lines from our null mice. However, we are hampered in this by the extremely restricted pattern of DUSP9/MKP-4 expression. Primary mouse embryo fibroblasts derived from wild-type animals do not express detectable DUSP9/MKP-4 protein, nor do embryonic kidney cells in culture (our unpublished observations). Although DUSP9/MKP-4 is expressed in mouse ES cells, these cells also express significant levels of the closely related phosphatases DUSP6/MKP-3 and DUSP7/MKP-X, with DUSP9/MKP-4 null ES cells displaying no apparent abnormalities in either the levels or phosphorylation of ERK1/2 or p38 MAPKs. One possible approach which might prove useful in future studies would be to derive trophoblast stem cells from wild-type and MKP-4 null embryos as described by Tanaka et al. (31). These cells can be cultured indefinitely in the presence of FGF4, can express specific markers of the extraembryonic ectoderm, and can be used to study the ability to differentiate into trophoblast giant cells in vitro.

In support of a role for DUSP9/MKP-4 in regulating MAPK activities in the placenta, the defects we find are strikingly similar to those observed following deletion of either MEK1, an upstream activator of ERK1/2, or the stress-activated p38α MAPK (1, 12). In both cases, the labyrinth layer was much thinner, with an almost complete lack of intermingling of embryonic and maternal blood vessels. Thus, while we cannot absolutely rule out the involvement of either a non-MAPK target or another MAPK isoform such as ERK5 in mediating the developmental defects we observe on deletion of DUSP9/MKP-4, it is highly likely that abnormal regulation of either or both of the ERK1/2 and p38α MAPK pathways is involved.

Given the very specific and robust expression of DUSP9/MKP-4 at several sites in mouse embryos and in particular in the developing liver (6), it was somewhat surprising that the rescue of defects in the extraembryonic tissues allows DUSP9/MKP-4 null embryos to develop to term, giving rise to apparently normal animals. One clue comes from an analysis of the representation of DUSP6/MKP-3, DUSP7/MKP-X, and DUSP9/MKP-4 in a variety of model organisms for which either the entire genome and/or transcriptome has been subjected to comprehensive sequence analysis. Extensive BLAST searching of the vertebrate sequence databases reveals that although definitive orthologues of DUSP6/MKP-3 and DUSP7/MKP-X are evident in all vertebrates (including fish, avian, amphibian, and mammalian organisms), sequences

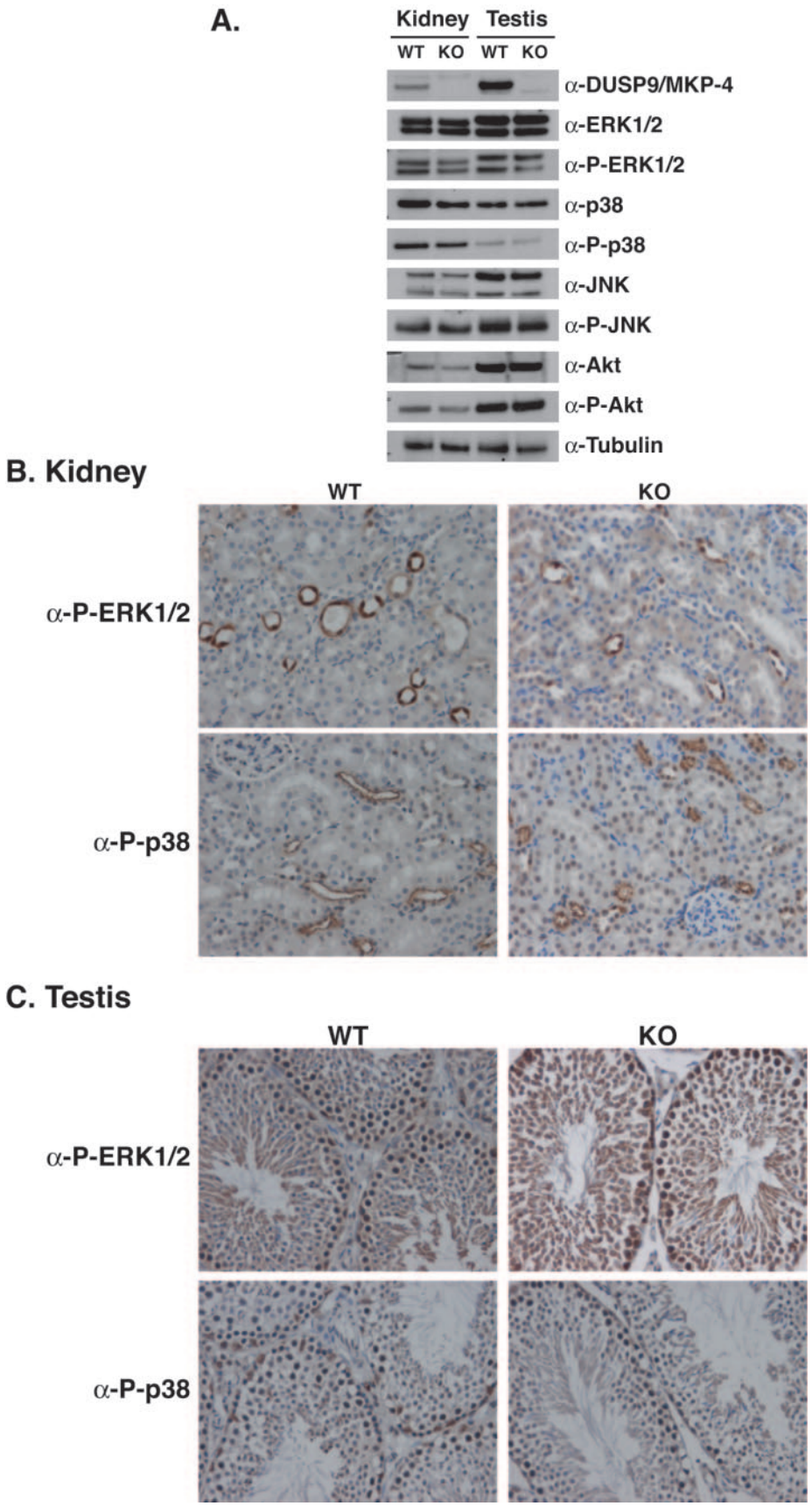

FIG. 6. Loss of DUSP9/MKP-4 does not lead to significant changes in the basal phosphorylation state of MAPKs in adult kidney or testis tissue. (A) Western blot analysis of protein lysates from wild-type (WT) or null (KO) kidney and testis tissues. The protein species detected are indicated on the right, and tubulin is included (bottom) as a loading control. (B) Representative sections from WT (left) and KO (right) adult kidney tissue stained using antibodies recognizing the phosphorylated (active) form of ERK1/2 (top) or p38 MAPK (bottom). (C) Representative sections from WT (left) and KO (right) adult testis tissue stained using antibodies against the phosphorylated (active) form of ERK1/2 (top) or p38 MAPK (bottom).

bearing hallmarks characteristic of DUSP9/MKP-4 were only identified in placental mammals. This might be explained by nondetection of a rare transcript in organisms for which only expressed sequence tag data were available. However, the completed genomes of the zebra fish *Danio rerio*, the puffer fish *Takifugu rubripes*, and the chicken *Gallus gallus* completely lack any sequence for a DUSP9/MKP-4 orthologue.

In view of the knockout phenotype and the location of DUSP9/MKP-4 on the X chromosome, which is known to feature genes important for placental development (15), we propose that DUSP9/MKP-4 has arisen since the divergence of mammals from other vertebrate lineages, acquiring an essential role in placentogenesis while being dispensable for the development of other organs such as the liver. It will be of interest to know if DUSP9/MKP-4 is represented in noneutherian mammals such as marsupials and monotremes as these develop in the absence of definitive placental structures such as the labyrinth and instead feature primitive chorionic and allantoic structures more similar to avian and reptilian structures (11, 17). We await confirmation from the ongoing marsupial genome project as to whether DUSP9/MKP4 is represented in those organisms.

Finally, there is little or no overlap of the expression patterns of DUSP9/MKP-4 with either DUSP6/MKP-3 or DUSP7/MKP-X, particularly in the developing liver (5, 6). This makes simple functional redundancy with respect to these closely related enzymes unlikely. However, we cannot rule out the possibility that other MKPs, which are normally either inactive or not expressed, might become upregulated, thus avoiding developmental abnormalities in tissues lacking DUSP9/MKP-4. Targeted inactivation of additional dual-specificity MKPs will help to elucidate whether other phosphatases can compensate for the lack of DUSP9/MKP-4 during mouse development.

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