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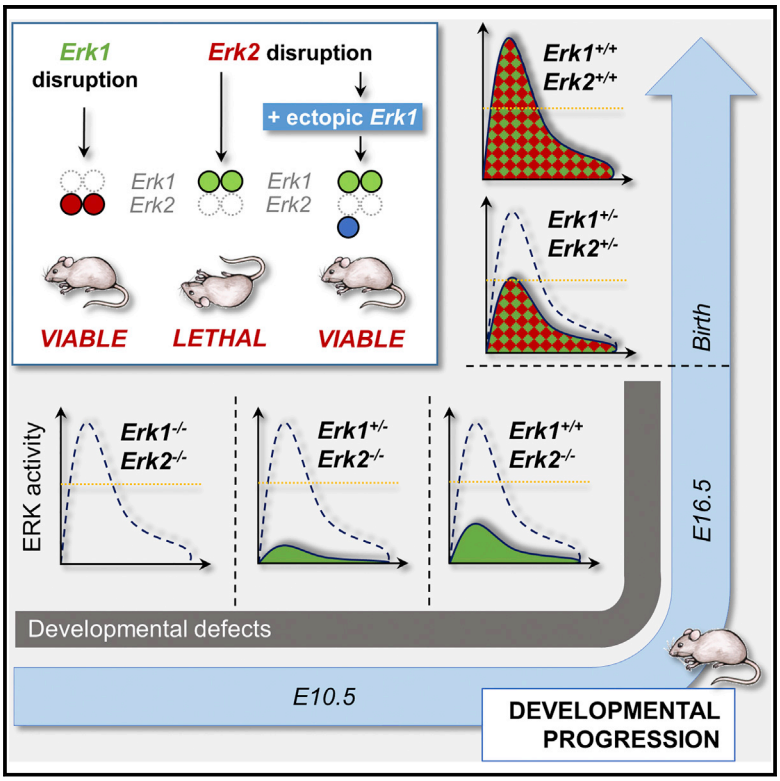
Functional Redundancy of ERK1 and ERK2 MAP Kinases during Development

Graphical Abstract



Highlights

- Genetic inactivation of *Erk2* in the embryo leads to severe developmental defects
- Developmental outcome dose-dependently correlates with global ERK1/2 activity
- Transgenic ERK1 rescues all developmental defects resulting from the loss of ERK2
- ERK1-only mice are viable, morphologically normal, and fertile

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In Brief

ERK1 and ERK2 are the effector kinases of the evolutionarily conserved ERK1/2 MAP-kinase signaling pathway. Using genetic approaches, Frémin et al. demonstrate that ERK1 and ERK2 isoforms exert redundant functions during mouse development. Transgenic expression of ERK1 fully rescues the developmental defects associated with the loss of ERK2.

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Functional Redundancy of ERK1 and ERK2 MAP Kinases during Development

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SUMMARY

ERK1 and ERK2 are the effector kinases of the ERK1/2 MAP-kinase signaling pathway, which plays a central role in transducing signals controlling cell proliferation, differentiation, and survival. Deregulated activity of the ERK1/2 pathway is linked to a group of developmental syndromes and contributes to the pathogenesis of various human diseases. One fundamental question that remains unaddressed is whether ERK1 and ERK2 have evolved unique physiological functions or whether they are used redundantly to reach a threshold of global ERK activity. Here, we show that the extent of development of the mouse placenta and embryo bearing different combinations of *Erk1* and *Erk2* alleles is strictly correlated with total ERK1/2 activity. We further demonstrate that transgenic expression of ERK1 fully rescues the embryonic and placental developmental defects associated with the loss of ERK2. We conclude that ERK1 and ERK2 exert redundant functions in mouse development.

INTRODUCTION

The ERK1/2 MAP kinase pathway is an evolutionarily conserved signaling module that processes information from a wide spectrum of extracellular stimuli to regulate cellular responses such as proliferation, survival, differentiation, and migration (Chang and Karin, 2001; Pearson et al., 2001). Perturbation of ERK1/2 signaling as a result of germline gain-of-function or loss-of-function mutations in pathway components is causally linked to a group of human congenital disorders characterized by facial dysmorphism, cardiac malformations, cutaneous and musculoskeletal abnormalities, and cognitive impairment (Rauen, 2013; Samuels et al., 2009). The ERK1/2 pathway is also commonly deregulated in human cancers and in various other diseases (Frémin and Meloche, 2010; Lawrence et al., 2008).

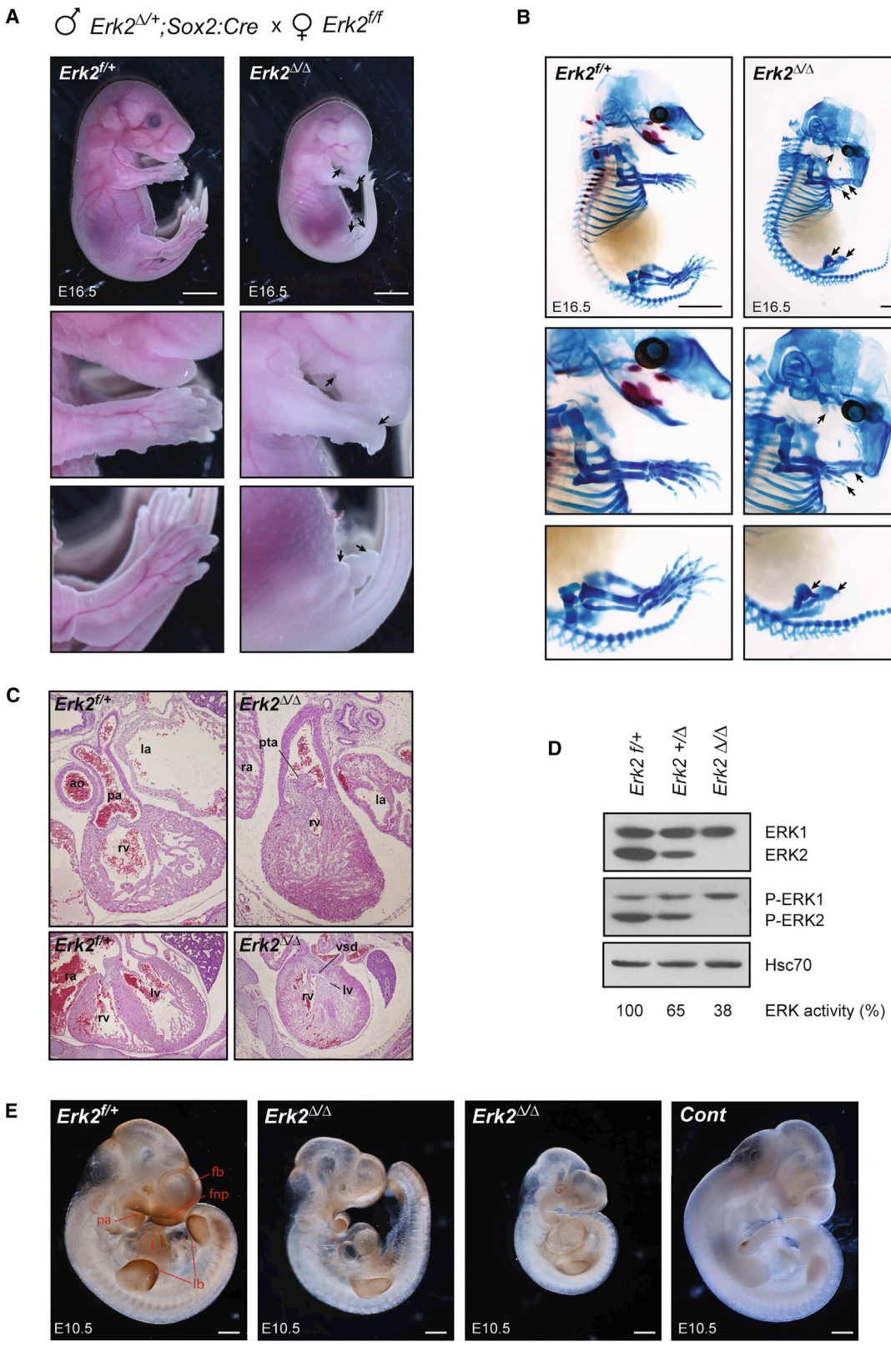
In mammals, signaling by the ERK1/2 pathway is relayed by the effector kinase isoforms ERK1 and ERK2, which share 83% amino acid identity (Boulton et al., 1990, 1991). Despite an abundant literature on this pathway, it is not known whether ERK1 and ERK2 have evolved specific physiological functions or whether they are used redundantly. *Erk1*$^{-/-}$ mice are viable and display no overt phenotype (Pagès et al., 1999), whereas *Erk2*$^{-/-}$ mice die in utero around embryonic day (E) 6.5, despite expression of ERK1 in early embryos (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003). Various mouse studies have reported that disruption of *Erk1* or *Erk2* results in distinct phenotypic consequences, arguing for isoform-specific functions of the two kinases (Bost et al., 2005; Bourcier et al., 2006; Guihard et al., 2010; Samuels et al., 2009; Satoh et al., 2007). On the other hand, additional inactivation of *Erk1* was found to aggravate the neural crest and neurogenesis defects of ERK2-deficient mice, suggesting a gene dosage-dependent contribution of ERK1/2 signaling (Newbern et al., 2008; Satoh et al., 2011). In vitro studies of the individual roles of ERK1 and ERK2 in cellular proliferation and transformation also yielded conflicting conclusions (Bourcier et al., 2006; Lefloch et al., 2008; Shin et al., 2010; Vantaggiato et al., 2006; Voisin et al., 2010).

In this study, we used a combination of genetic approaches to rigorously address the functions of ERK1 and ERK2 isoforms in mouse embryonic development. We generated embryos carrying different combinations of *Erk1* and *Erk2* alleles and established that the extent of embryonic and placental development is dependent on the global ERK1/2 activity. Importantly, we showed that transgenic overexpression of ERK1 rescues the placental and embryonic phenotypes of ERK2-deficient mice and the proliferation defects of ERK1/2-deficient mouse fibroblasts. Mice expressing exclusively ERK1 are viable, fertile, and morphologically normal. Our results provide compelling evidence for a functionally redundant role of ERK1 and ERK2 MAP kinases during development.

RESULTS AND DISCUSSION

Disruption of *Erk2* in the Whole Embryo Leads to Severe Developmental Defects

The early lethality of ERK2-deficient embryos, consequent to a cell-autonomous defect in trophoblast development (Saba-El-Leil et al., 2003), precludes analysis of ERK2 functions in development. To circumvent this problem, we generated a conditional



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allele of *Erk2* and confirmed that the floxed (flox) and Cre-excised deletion (Δ) alleles behave like wild-type and null alleles, respectively (Figure S1). To investigate the impact of *Erk2* deficiency on the embryo per se, we crossed $Erk2^{flox/flox}$ mice with Sox2:Cre transgenic mice (Hayashi et al., 2002) to inactivate *Erk2* exclusively in the epiblast and bypass extra-embryonic defects. No viable $Erk2^{\Delta/\Delta}$ embryo was detected at E18.5 (0 of 41), but some embryos survived up to E16.5 (Table S1). However, these embryos exhibited major craniofacial abnormalities, including a lack of mandible and tongue, shortened maxilla, and cardiovascular malformations such as ventricular septal defects and persistent truncus arteriosus (Figures 1A–1C). These phenotypes are indicative of impaired neural crest development, as previously reported (Newbern et al., 2008). In addition, $Erk2^{\Delta/\Delta}$ embryos were smaller and, most notably, had short forelimbs and lacked hindlimbs (Figures 1A and 1B). Complete excision of the *Erk2* gene was confirmed on E10.5 embryos (Figure 1D). As previously observed by us and others, loss of *Erk2* alleles does not result in compensatory expression of ERK1 protein. Interestingly, the anatomical location of these morphological defects was closely related to areas displaying strong ERK1/2 activity during development: frontonasal process, forebrain, pharyngeal arches, limb buds, and tailbud (Figure 1E; Corson et al., 2003). As expected, ERK2-deficient embryos showed a marked decrease of ERK1/2 activity at these sites.

Development of the Embryo and Placenta Is Dependent on Total ERK1/2 Activity

We then investigated the effect of ERK1/2 gene dosage on embryonic development by comparing the phenotypes of E14.5 placentas and embryos bearing different combinations of *Erk1* and *Erk2* alleles. We could establish a close correlation between the total ERK1/2 activity in the placenta and its development (Figures 2A–2D). Loss of a single *Erk2* allele significantly decreased the placental weight (Figure 2B) and surface area of the labyrinth (Figures 2A and 2C). Additional disruption of *Erk1* alleles exacerbated these effects. ERK2 was estimated to contribute to 75%–80% of the global ERK1/2 activity in the placenta (Figures 2D, S2B, and S2C). Close examination of the placental architecture revealed no obvious differences among different genotypes other than the reduced size and surface area of the labyrinth layer, which forms a critical interface between maternal and fetal circulation, facilitating nutrient exchange and thereby impacting directly on embryonic growth (Figure S2A). Many mouse mutants with a placental phenotype are characterized by structural defects in the formation of the labyrinth layer, resulting in reduced exchange of nutrients and fetal growth restriction. These include ERK1/2 (Hatano et al., 2003; this study) and their upstream kinase MEK1 (Giroux et al., 1999). Notably, embryos with the same *Erk1/2* alleles but developing from placentas carrying different allelic combination have distinct weights (Figures S2D–S2L), emphasizing the importance of the role of placental ERK1/2 activity in controlling embryonic growth.

The extent of embryonic development was also related to total ERK1/2 activity. Embryos with two *Erk2* alleles were similar in size and weight irrespective of ERK1 activity (Figures 2E–2G). Embryos with one *Erk2* allele were morphologically normal, but their weight decreased proportional to the number of *Erk1* alleles. Specifically, we noted a progressive delay in the emergence of digits during limb development with the gradual reduction in ERK1/2 activity (Figures 2E and 2G). Animals with a single allele of *Erk2* ($Erk1^{-/-}$;$Erk2^{\Delta/+}$) were born but die within the first hours of life. Finally, embryos with one *Erk1* allele ($Erk1^{+/-}$; $Erk2^{\Delta/\Delta}$) were very small and their development stopped prematurely at E10.5 (Figure S2M). These results reveal a tight relationship between ERK1/2 protein dosage and developmental outcome, suggesting that ERK1 and ERK2 act redundantly during embryonic development.

Transgenic Expression of ERK1 Rescues Embryonic and Placental Defects Associated with the Loss of ERK2

To further address this question, we next asked whether ERK1 could substitute for ERK2 in mouse development. We first generated knockin mouse models in which the mouse *Erk1* or *Erk2* (as control) cDNA sequence was inserted into exon 1 of the *Erk2* gene. However, both *Erk1* and *Erk2* knockin alleles were expressed at much lower levels than endogenous *Erk2*, precluding the use of these mutant mice for quantitative phenotypic analyses. As an alternative strategy, we generated a transgenic mouse that ubiquitously expresses ERK1 from the chicken beta-actin promoter (Figure S3A). This ubiquitous promoter directs constitutive transgene expression during all developmental stages, from unfertilized eggs onward, and in adult tissues (Okabe et al., 1997). We showed that TgERK1 mRNA (Figure S3B) and protein (Figure S3C) were expressed at all developmental stages examined, from E10.5 to E18.5. Expression of TgERK1 did not affect the expression level of ERK2 isoform. *TgErk1* mice were crossed to $Erk2^{\Delta/+}$ heterozygous mice, and the F1 $Erk2^{\Delta/+}$;*TgErk1* progeny was intercrossed to generate $Erk2^{\Delta/\Delta}$;*TgErk1* mice. At E14.5, whereas $Erk2^{\Delta/\Delta}$ embryos were never found in litters, $Erk2^{\Delta/\Delta}$;*TgErk1* embryos were recovered in Mendelian proportions (Table S2). $Erk2^{\Delta/\Delta}$;*TgErk1* embryos were morphologically indistinguishable from $Erk2^{+/+}$ embryos

Figure 1. Targeted Disruption of *Erk2* in the Epiblast Leads to Severe Morphological Defects

$Erk2^{\Delta/+}$;*Sox2:Cre* male mice were crossed with female $Erk2^{flox/flox}$ mice.

(A) Phenotypic appearance of embryos at E16.5. $Erk2^{\Delta/\Delta}$ embryos exhibit a smaller size, shortened maxilla, lack of mandible, short forelimbs, and absence of hindlimbs (indicated by arrows). The scale bar represents 3 mm.

(B) Alizarin red/Alcian blue staining of E16.5 embryos. The scale bar represents 3 mm.

(C) H&E staining of cross-sections of E16.5 hearts showing ventricular septal defects (vsd) and persistent truncus arteriosus (pta) in $Erk2^{\Delta/\Delta}$ embryos. ao, aorta; la, left atrium; lv, left ventricle; pa, pulmonary artery; ra, right atrium; rv, right ventricle.

(D) Immunoblot analysis of ERK1/2 expression and activity in extracts from E10.5 embryos.

(E) Whole-mount immunohistochemistry analysis of phosphorylated ERK1/2 in E10.5 embryos. Strong ERK1/2 activity localizes to frontonasal process (fnp), pharyngeal arches (pa), forebrain (fb), liver primordia (l), and limb buds (lb). Cont, control without primary antibody. The scale bar represents 300 μm.

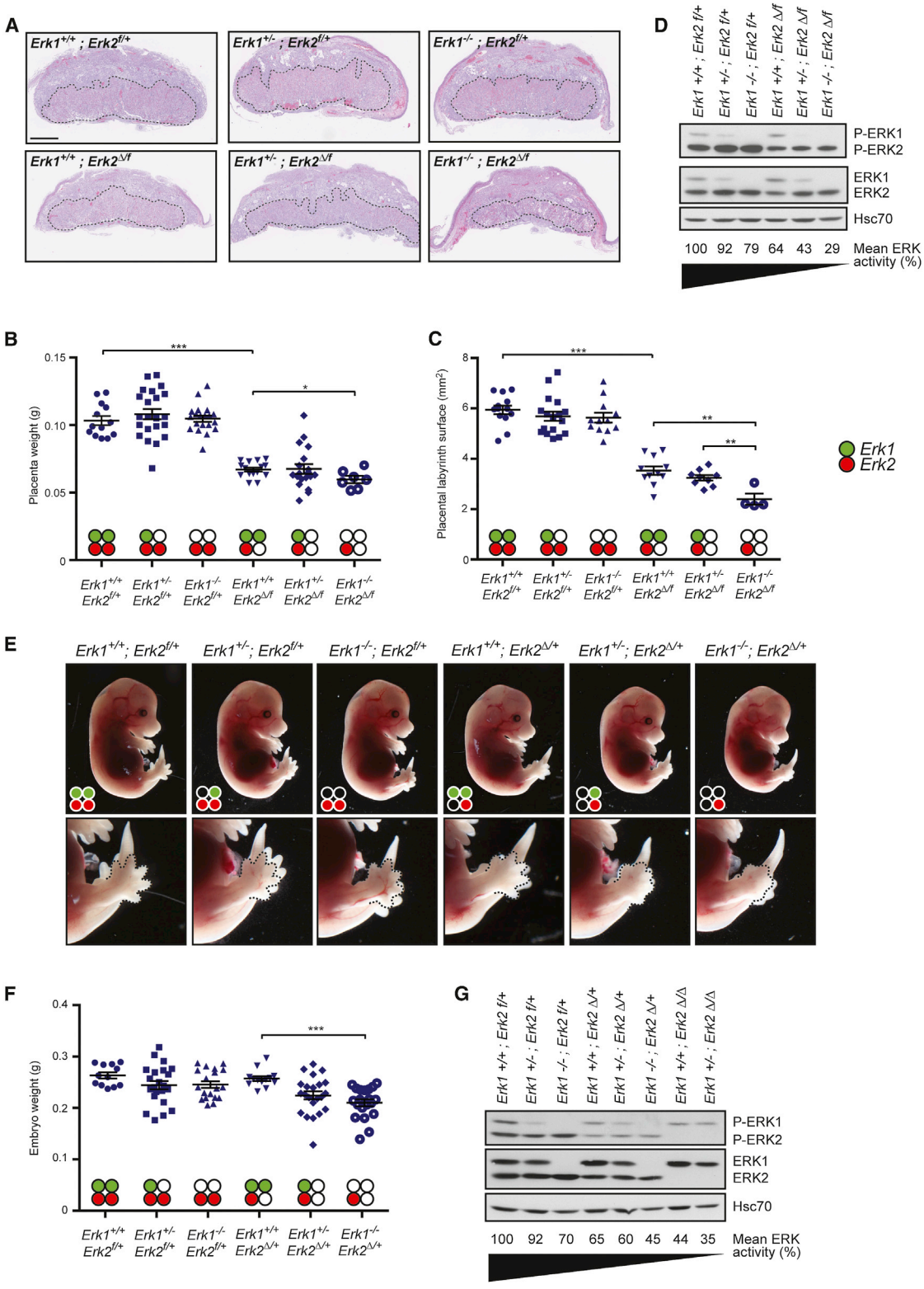

Figure 2. The Extent of Placental and Embryonic Development Is Dependent on Global ERK1/2 Activity

Two types of crosses were performed to obtain placentas and embryos of the desired genotypes. *Erk2*$^{\Delta/+}$;*Sox2*:*Cre* males were crossed with *Erk2*$^{flox/flox}$ females, and *Erk1*$^{+/-}$;*Erk2*$^{\Delta/+}$;*Sox2*:*Cre* males were crossed with *Erk1*$^{-/-}$;*Erk2*$^{flox/flox}$ female mice.

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throughout embryonic development, from E10.5 to birth (Figure 3A), and had comparable weight (Figure 3B). Craniofacial and limb defects resulting from absence of ERK2 were completely rescued, as well as cardiac malformations (Figure S3D). *TgErk1* restored the decrease in placental weight observed upon the loss of one *Erk2* allele (Figure 3C). Notably, the architecture and weight of $Erk2^{\Delta/\Delta}$;*TgErk1* placentas were identical to that of $Erk2^{+/+}$ placentas (Figures 3C and 3D). We confirmed by quantitative immunoblotting that ectopic expression of TgERK1 restores normal global ERK1/2 activity in $Erk2^{\Delta/\Delta}$;*TgErk1* embryos and placentas (Figures 3E and 3F). Importantly, the spatial pattern of ERK signaling in intact $Erk2^{\Delta/\Delta}$ TgERK1-expressing embryos was similar to that of phenotypically normal embryos expressing one or two alleles of Erk2 (Figures S3E and 1E).

Mice Overexpressing Exclusively ERK1 Isoform Are Viable, Morphologically Normal, and Fertile

Adult $Erk2^{\Delta/\Delta}$;*TgErk1* animals were also found in Mendelian proportions (Table S2) and appeared morphologically normal (Figure 4A). Whereas $Erk2^{\Delta/+}$ mice are significantly smaller than wild-type mice, $Erk2^{\Delta/\Delta}$;*TgErk1* mice had a normal weight (Figure 4B). Macroscopic examination of organs from $Erk2^{\Delta/\Delta}$;*TgErk1* animals at 8 weeks did not reveal any abnormality (Figure S3F). Detailed histological analyses further confirmed the normal architecture of tissues (Figure S3G). No difference could be observed in the localization and extent of phospho-ERK1/2 activity in tissues from $Erk2^{+/+}$ and $Erk2^{\Delta/\Delta}$;*TgErk1* animals (Figure 4C). TgERK1 expression was detected in all tissues examined (Figure S3H). It is noteworthy that the ERK1 transgene was expressed at high levels in several tissues, such as muscle, pancreas, heart, kidney, or stomach, but that the global ERK1/2 activity was comparable between $Erk2^{+/+}$ and $Erk2^{\Delta/\Delta}$;*TgErk1* tissues (Figure S3H). This indicates that the steady-state activation of ERK1/2 is very robust against perturbations of ERK protein levels, confirming previous findings obtained in cultured cell lines (Fritsche-Guenther et al., 2011). Whereas multiple post-translational mechanisms may contribute to the observed robustness, analysis of in vitro cellular models suggests that negative feedback to RAF plays a major role in conferring robustness to long-term ERK1/2 signaling (Fritsche-Guenther et al., 2011). Our results demonstrate the robustness of ERK1/2 signaling at the organismal level.

$Erk2^{\Delta/\Delta}$;*TgErk1* animals exhibited no overt phenotype after 12 months. We also tested whether these mice are fertile. Intercrossed $Erk2^{\Delta/\Delta}$;*TgErk1* animals were able to reproduce and gave rise to normal progeny (Figures 4D and 4E). Finally, we intercrossed $Erk1^{+/-}$;$Erk2^{\Delta/\Delta}$;*TgErk1* mice and generated viable and morphologically normal $Erk1^{-/-}$;$Erk2^{\Delta/\Delta}$;*TgErk1* adult animals, thus demonstrating that TgERK1 is able to compensate for both endogenous ERK1 and ERK2 proteins (Table S3). Altogether, our results demonstrate that overexpression of ERK1 can compensate for the absence of ERK2 in $Erk2^{-/-}$ mice.

We also analyzed the ability of TgERK1 to rescue the proliferative defects of ERK1/2 mutant mouse embryonic fibroblasts (MEFs) in vitro (Voisin et al., 2010). $Erk2^{\Delta/\Delta}$;*TgErk1* MEFs proliferated at similar rate than wild-type MEFs, whereas $Erk2^{\Delta/\Delta}$ MEFs showed slower proliferation as previously documented (Figure S4A). Importantly, expression of TgERK1 completely rescued the proliferation arrest and senescence induction observed in ERK1/2 double-knockout MEFs (Voisin et al., 2010). TgERK1 was activated with similar kinetics as endogenous ERK1 and ERK2, and its expression fully restored the normal phosphorylation of a panel of ERK1/2 substrates (Figure S4B). These included Fra-1, which has been described as an ERK2-specific target (Shin et al., 2010). Similarly, expression of TgERK1 conferred normal transcriptional regulation of ERK1/2 target genes upon EGF stimulation (Figure S4C).

The question of ERK1 and ERK2 functional redundancy has been a subject of intense research and controversy in the signal transduction field. Several studies have argued for specific roles of ERK1 and ERK2 MAP kinases based on the genetic inactivation of one of the two isoforms by homologous recombination or RNAi in cultured cells and mice (Bost et al., 2005; Bourcier et al., 2006; Frémin et al., 2007; Guihard et al., 2010; Radtke et al., 2013; Satoh et al., 2007; Shin et al., 2010; Vantaggiato et al., 2006). However, these studies did not take into account the global ERK1/2 activity in the analysis of the phenotypes resulting from *Erk1* or *Erk2* inactivation. This aspect is crucial as ERK1 and ERK2 are differently expressed in many cell lines and tissues, and ERK2 is the predominantly expressed isoform in most mouse tissues (Figure S3H). Our study now provides compelling genetic evidence for a functionally redundant role of ERK1 and ERK2 MAP kinases. By highlighting the close relationship between the extent of embryonic development and the global level of ERK1/2 activity, we propose that a threshold of ERK1/2 activity determines normal developmental progression (Figure 4F). The two redundant isoforms of ERK1/2 provide a pool of kinases available for activation, and different thresholds of activity are required for executing different developmental decisions in specific tissues. Because ERK2 is the predominantly expressed kinase in the mouse, loss of ERK2 results in a larger spectrum of phenotypes that can be rescued simply by increasing ERK1 expression as demonstrated here. However,

(A) Placental sections of E14.5 embryos were made at the chorioallantoic region and stained with H&E to visualize the labyrinth architecture (dotted line). The scale bar represents 1 mm.
(B and C) The weight (B) of E14.5 placentas and the surface of sectioned labyrinth (C) were measured.
(D) Immunoblot analysis of ERK1/2 in extracts from E14.5 placentas. A representative blot is shown. Quantification of ERK activity was performed on three individual placentas.
(E) Phenotypic appearance of embryos of the indicated genotypes. Bottom panels, detail of developing toes.
(F) Weight of E14.5 embryos.
(G) Immunoblot analysis of ERK1/2 in extracts from E10.5 embryos. A representative blot is shown. Quantification was performed on three individual embryos. *Erk1* and *Erk2* genes are represented by green and red circles, respectively. Wild-type and null alleles are symbolized by filled and empty circles. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

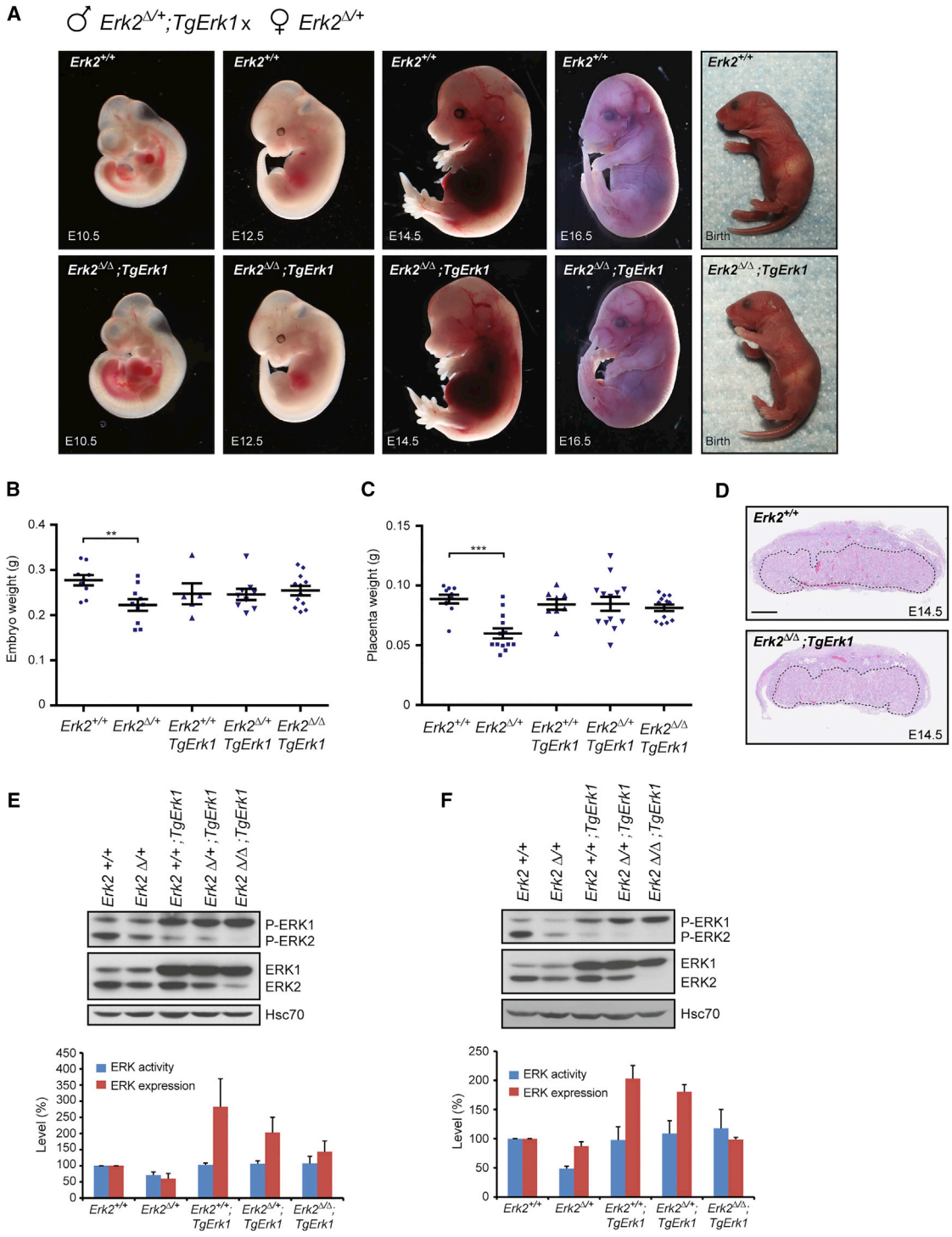

Figure 3. Transgenic Expression of ERK1 Rescues the Developmental Defects of ERK2-Deficient Embryos

Erk2$^{\Delta/+}$ mice were crossed with *Erk2*$^{\Delta/+}$;*TgErk1* mice.

(A) Phenotypic appearance of embryos from E10.5 to E16.5 and newborn mice.

(B) Weight of E14.5 embryos.

(C) Weight of E14.5 placentas.

(D) H&E staining of sagittal sections from E14.5 placentas. The scale bar represents 1 mm.

(E and F) Immunoblot analysis of ERK1/2 in extracts from E14.5 placentas (E) and E10.5 embryos (F). Quantification of ERK1/2 activity and expression was performed on three individual samples and is reported as mean ± SEM. The value of wild-type mice was set at 100%. **p < 0.01; ***p < 0.001.

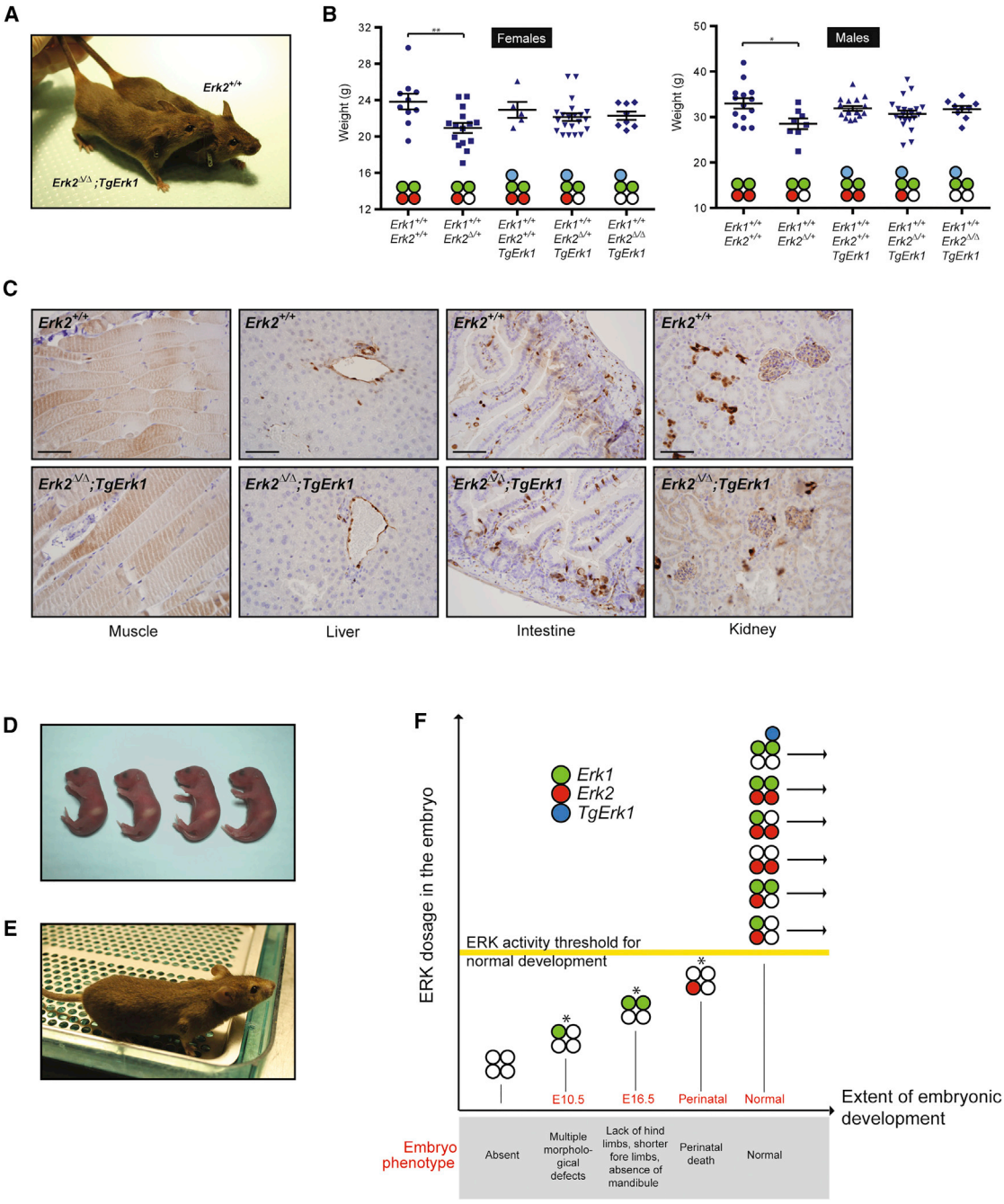

Figure 4. Characterization of *Erk2*$^{\Delta/\Delta}$;*TgErk1* Mice

Erk2$^{\Delta/+}$ mice were crossed with *Erk2*$^{\Delta/+}$;*TgErk1* mice.

(A) Phenotypic appearance of *Erk2*$^{\Delta/\Delta}$;*TgErk1* mice at 8 weeks.

(B) Weight of 8-week-old female and male mice. *$p < 0.05$; **$p < 0.01$.

(C) Immunohistochemical staining for phospho-ERK1/2 in muscle, liver, intestine, and kidney. The scale bar represents 50 μm.

(D and E) *Erk2*$^{\Delta/\Delta}$;*TgErk1* mice were intercrossed and gave birth to normal progeny. Phenotypic appearance of newborn (D) and 8-week-old adult (E) mice.

(F) Schematic diagram of the extent of mouse embryonic development as a function of ERK1/2 gene dosage. Blue circle represents *TgErk1* transgene. *These embryonic phenotypes are only observed upon epiblast-specific inactivation of *Erk2* to bypass extra-embryonic defects. Normal embryonic development requires a minimal threshold of ERK1/2 activity that we estimate to be the sum of one allele of *Erk1* and *Erk2*.

we cannot definitely exclude that ERK1 and ERK2 exert non-redundant functions in adult animals, in a normal or pathological context.

The implications of our findings are important and wide ranging. The ERK1/2 MAP kinase pathway is a key regulator of several cellular responses, but a comprehensive picture of its physiological functions has been hampered in many studies by a lack of understanding of the individual roles of the two ERK isoforms. Most importantly, deregulation of the ERK1/2 pathway is causally linked to a group of human congenital disorders known as RASopathies and to cancer. Small-molecule inhibitors of BRAF and MEK1/2 have been approved for the treatment of metastatic melanoma, and the first inhibitor of ERK1/2 has just entered into clinical evaluation. Because all these compounds are assumed to exert their clinical activity mainly through inhibition of their downstream effectors ERK1 and ERK2, it is crucial to understand whether the two isoforms exert specific or redundant functions.

EXPERIMENTAL PROCEDURES

Manipulation of Mice

All mice were bred under standard conditions at the Institute for Research in Immunology and Cancer. Mice were housed under specific pathogen-free conditions in filter-topped isolator cages under a 12/12 hr light/dark cycle with access to food and water ad libitum. Animals were handled in strict accordance with good animal practice as defined by the relevant local animal welfare bodies, and all experiments were approved by the Canadian Council on Animal Care (CCAC).

Gene Targeting and Generation of Transgenic Strains

We generated a conditional *Erk2* allele by flanking exon 3 of the *Erk2* gene with *loxP* sites (Figure S1). The targeting vector was linearized and electroporated into G4 ESCs. Recombinant clones were selected with G418 and screened by Southern blot analysis for homologous recombination. Correctly targeted ESC clones were injected into C57BL/6 blastocyst-stage embryos to produce chimeric mice, and germline transmission was obtained from two independent clones. For the conditional *Erk2* allele, the floxed neomycin resistance cassette was removed by mating $Erk2^{floxneo/+}$ male with *Zp3*:*Cre* female mice that express Cre recombinase in growing oocytes.

The *TgErk1* construct was obtained by cloning the full-length mouse ERK1 cDNA into the *EcoR*I site of pCAGGS vector containing the cytomegalovirus enhancer, chicken beta-actin promoter, beta-actin intron, and a rabbit β-globin polyadenylation signal (Okabe et al., 1997). pCAGGS was kindly provided by F. Charron (Université de Montréal). The SalI-*Psi*I linearized transgenic construct (3.7 kb) was microinjected into the pronucleus of FVB/N fertilized oocytes, and the resulting embryos were implanted into pseudopregnant mice. Mice expressing the *TgErk1* transgene were identified by PCR analysis. Two independent founder lines were selected and bred with CD1 mice.

All the strains were maintained in a mixed CD1 genetic background. For genotyping analysis, genomic DNA from embryos or tails was isolated by proteinase K treatment in SDS buffer.

Histological and Immunohistochemistry Methods

For staining with H&E, embryos, placentas, and tissues were fixed in formalin for at least 48 hr, embedded in paraffin, sectioned, and stained according to standard procedures. To calculate the labyrinth surface area, placentas were sectioned longitudinally at the chorioallantoic region, scanned, and analyzed using The Nanozoomer Digital Pathology (NDP) system (Hamamatsu). For staining of bone and cartilage, embryos were fixed in ice-cold 95% ethanol overnight and then stained with 0.02% Alcian Blue and 0.005% Alizarin Red in 5% acetic acid and 70% ethanol for 24 hr. Soft tissues were cleared in 1% KOH for 24 hr, and the samples were incubated in solutions of increasing concentration of glycerol according to standard protocols.

Whole-mount immunohistochemistry analysis of phospho-ERK1/2 was performed as described previously (Corson et al., 2003). Briefly, E10.5 embryos were rapidly dissected in ice-cold PBS and transferred immediately to cold 8% paraformaldehyde overnight to preserve endogenous ERK1/2 signaling. Embryos were dehydrated in methanol, bleached with 5% H_2O_2, rehydrated, blocked with BSA-TBST (5% BSA in TBS [10 mM Tris-HCl (pH 7.5), 150 mM NaCl] and 0.1% Triton X-100), and incubated with primary anti-phospho-ERK1/2 antibody (1:250) overnight. The embryos were washed six times, incubated with biotinylated secondary antibody overnight, washed again six times, and incubated with Vectastain ABC peroxidase kit (Vector Laboratories). For standard immunohistochemistry, tissue sections were washed three times in TBS, quenched for 5 min in 10% methanol, 1% H_2O_2 in TBS, and permeabilized for 15 min in 0.2% Triton X-100/TBS. The sections were incubated with anti-phospho-ERK1/2 antibody (1:100) for 2 hr at 37°C. Tissue sections were then washed three times in TBS, incubated with biotinylated anti-IgG secondary antibody (1:250) for 2 hr at room temperature, washed three times, incubated with avidin-biotin complex (1:500; Vector Laboratories) for 1 hr, washed three times, and finally revealed in diaminobenzidine substrate solution for 30 min.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.011.

AUTHOR CONTRIBUTIONS

C.F., M.K.S.-E.-L., and S.M. designed the study, conceived the experiments, and analyzed the data. C.F., M.K.S.-E.-L., and K.L. performed the experiments. S.-L.A. contributed to the generation of $Erk2^{flox}$ mice. The manuscript was written by C.F. and S.M. with contribution by M.K.S,-E,-L.

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