Supporting Information

Klinger et al. 10.1073/pnas.0900919106

SI Materials and Methods

Gene Targeting Strategy. The cloning of the murine *Mapk6* gene has been reported previously (1). A targeting vector was designed to replace a 5-kb genomic fragment of *Mapk6* containing exons 2, 3, and 4 (encoding amino acids 1–289 of Erk3) by the *LacZ* gene containing a polyadenylation signal inserted in frame at the initiation codon. The neomycin resistance gene (*Neo*) was inserted in the targeting vector downstream of the *LacZ* gene in the antisense orientation to *Mapk6* transcription. The *LacZ-Neo* cassette was flanked by 4.5-kb of *Mapk6* genomic DNA on both sides. The targeting vector was linearized with *Not*I and electroporated into R1 ES cells (2). After selection with 180 μg/mL G418, homologous recombinants were screened by Southern blot analysis. DNA samples were digested with *Eco*R1 and probed with a 5′ external probe (*Kpn*I-*Xba*I genomic fragment). Homologous recombination was detected in 3% of the clones (7/214).

Generation of *Mapk6*-Deficient Mice and Genotyping. Two correctly targeted ES clones (103K3 and 127K3) were separately injected into C57BL/6 host blastocysts to generate chimeric animals. Male chimeras were bred with C57BL/6 females and both clones transmitted the mutant allele to the germ line, as verified by Southern blot analysis. Heterozygous F1 offspring were then intercrossed to obtain homozygous *Mapk6* mutants. The subsequent progeny was genotyped by PCR. *Mapk6* specific primers (forward 5′-GTTGCTCATAAACACAAACCTCC-3′ and reverse 5′-GGGATGGGTTATGTTTTCTAGAC-3′) amplify a 300-bp fragment corresponding to the 5′ intron-exon boundary of exon 3. The mutated allele was amplified using *LacZ* specific primers (forward 5′-TAAACCGACTACACAAATCAGCG-3′ and reverse 5′-GCAGCAGCAGACCATTTTCAATC-3′) resulting in a 600-bp product.

Quantitative RT-PCR. Total RNA was isolated from E18.5 lung tissue, purified using the RNeasy kit (Qiagen) and reverse transcribed, RNA was reverse transcribed in a final volume of 100 μL using the High Capacity cDNA Archive Kit with random primers (Applied Biosystems) as described by the manufacturer. Sense and antisense oligonucleotides (5′-ACTGGTAAAACCCTCTTTGCAG-3′ and 5′-GGAATCACGCTGAGAAGCTC-3′) specific for *Mapk6* were used for PCR using 2 μL cDNA sample (50 ng), 5 μL TaqMan PCR Master Mix (Applied Biosystems), 2 μM each primer, and 1 μM Universal TaqMan probe in a total volume of 10 μL. Real-time analysis of PCR product amplification was performed on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase was used as endogenous control.

Histology and Immunohistochemistry. Tissues from embryos and newborns were fixed overnight in 10% formalin, embedded in paraffin and sectioned at a 5-μm thickness. Tissue sections were stained using conventional hematoxylin/eosin protocol. Lung sections were also stained with PAS. For quantitative morphometry, PAS-positive cells, tissue area and saccular airspace were measured with the Image ProPlus software on 10 randomly selected micrographs.

Paraffin sections were deparaffinized and boiled in citric acid buffer (pH 6.0) for 20 min in a microwave oven, followed by a 20-min cool down period. Endogenous peroxidase activity was quenched by incubation with 0.3% H_2O_2 for 30 min at room temperature. The sections were then stained with the following primary antibodies: anti-T1α (Developmental Studies Hybridoma Bank; dilution 1:50), anti-proSP-C (Chemicon; dilution 1:1000), anti-Ki67 (Santa Cruz Biotechnology; dilution 1:50). After washing, the sections were incubated with biotinylated secondary antibodies (Vector; dilution 1:100). Antibody binding was detected with the Vectastain ABC kit (Vector) and DAB (Vector) or AEC (Vector) as substrate according to the manufacturer's instructions. Sections were counterstained with hematoxylin and scanned with a NanoZoomer slide scanner (Hamamatsu Photonics). Ki67-positive cells were counted on 10 randomly selected micrographs relative to tissue area using the Image ProPlus software.

Lung Instillation. E18.5 fetuses were dissected to remove the abdomen from the upper body part. The diaphragm was dissected out from the rib cage to allow maximal inflation of the lung. The lungs were inflated by injection of a 0.5× solution of OCT (Tissue-Tek) into the trachea using a 25G blunted needle, until the lungs filled completely the thoracic cavity. After inflation, the trachea was knotted with a surgical suture and the upper body-rib cage was immediately covered with OCT and frozen on dry ice. Cryosections of the samples (7-μm thick) were fixed in 4% paraformaldehyde and stained using standard hematoxylin/eosin protocol.

Ultrastructural Analysis. Lung tissues were fixed by immersion in 1% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4 °C. Tissues were then washed in phosphate buffer, dehydrated in a series of graded methanol solutions at decreasing temperature until −30 °C and embedded in Lowicryl K4M (CANEMCO) as described previously (3). Epon embedding was carried out after postfixation of the tissue with 1% osmium tetroxide for 1 h at 4 °C, and dehydration in ethanol and propylene oxide. Thin tissue sections (70 nm) were mounted on Parlodion and carbon-coated nickel grids. Specimens were examined on a Philips 410LS electron microscope (FEI Systems).

Measurement of IGF Levels. Blood samples were collected from E18.5 embryos by decapitation and centrifuged to extract serum. IGF-1 levels were measured by ELISA (Quantikine, mouse IGF-1 ELISA, R&D Systems) according to the manufacturer's instructions. IGF-2 levels were measured by ELISA (DuoSet ELISA Developmental System, mouse IGF-2, R&D Systems) according to the manufacturer's instructions.

Statistical Analysis. Results are expressed as mean ± SEM. Statistical analyses were performed using Student's *t* tests with a significance threshold set at $P < 0.05$.

1. Turgeon B, Lang BF, Meloche S (2002) The protein kinase ERK3 is encoded by a single functional gene: Genomic analysis of the ERK3 gene family. *Genomics* 80:673–680.
2. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA* 90:8424–8428.
3. Bendayan M (1995) Colloidal gold post-embedding immunocytochemistry. *Prog Histochem Cytochem* 29:1–159.

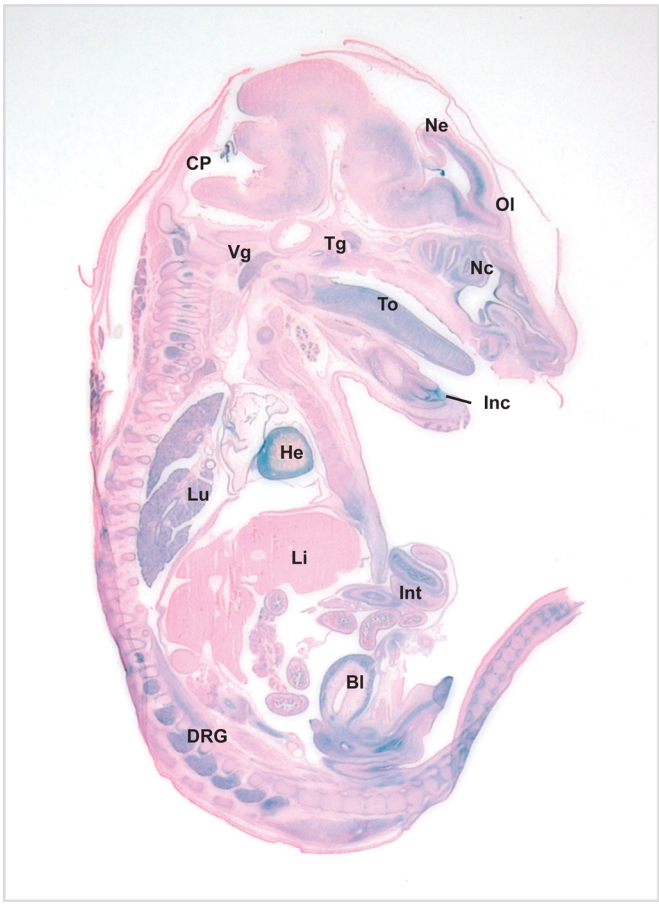

Fig. S1. Expression of *Mapk6*^{LacZ} during fetal development. The *Neo* gene was removed by crossing mice heterozygous for the targeted *Mapk6*^{LacZ}-*loxP*-flanked-*Neo* allele to CMV-Cre transgenic mice. Sagittal sections of E15.5 embryos were stained with X-gal to detect β-galactosidase activity. Abbreviations: Bl, bladder; CP, choroid plexus; Drg, dorsal root ganglion; H, heart; Inc, incisor; Int, intestine; Li, liver; Lu, lung; Ne, neocortex; Nc, nasal cavity; Ol, olfactory lobe; Vg, vagal (X) ganglion; Tg, trigeminal (V) ganglion; To, tongue.

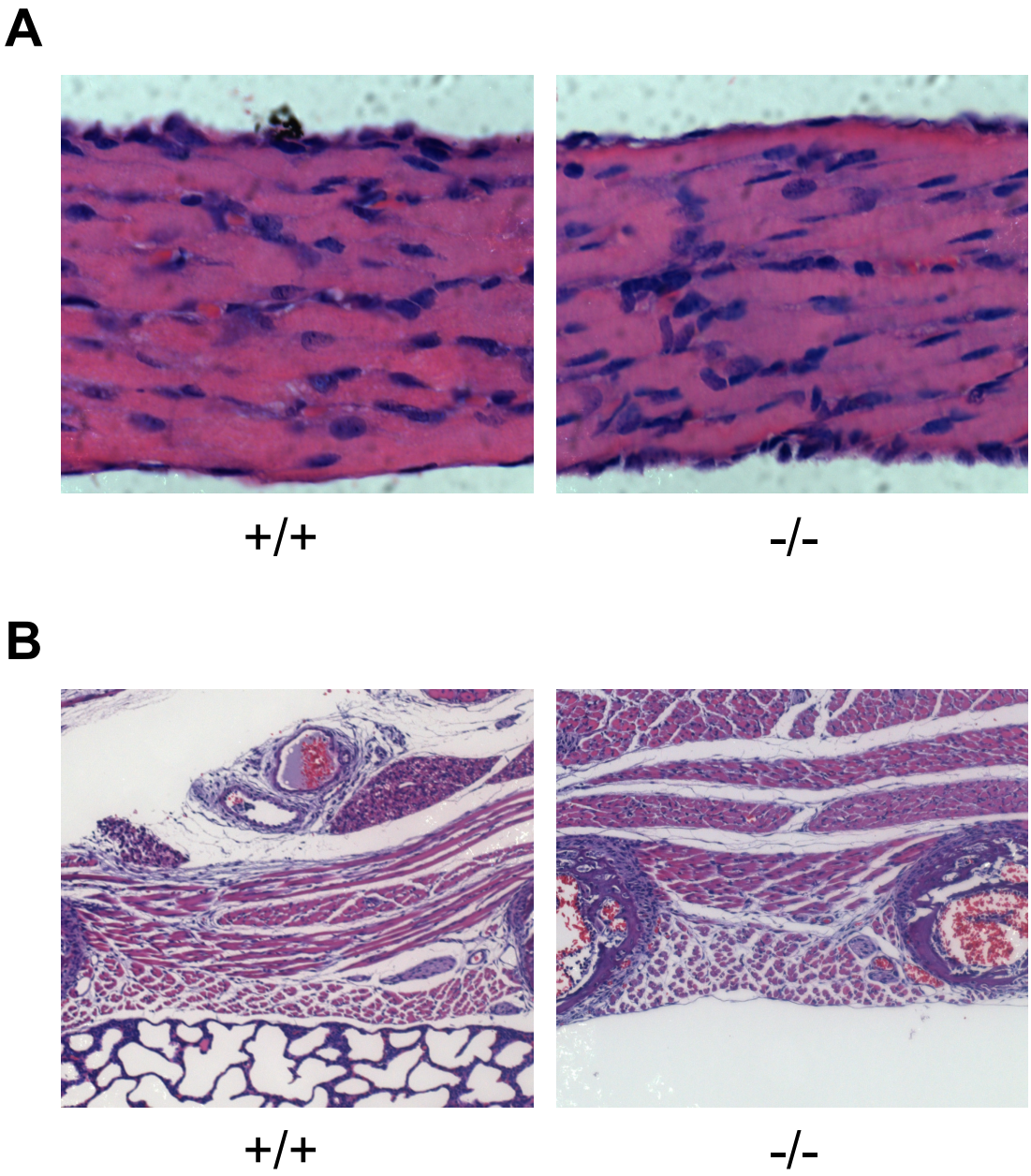

Fig. S2. Respiratory muscles of Erk3-deficient mice. Newborn Erk3$^{+/+}$ and Erk3$^{-/-}$ mice were fixed in formalin, embedded in paraffin and sectioned in the coronal plane. Sections were stained with hematoxylin and eosin. Diaphragm (*A*) and intercostal (*B*) muscles are shown.

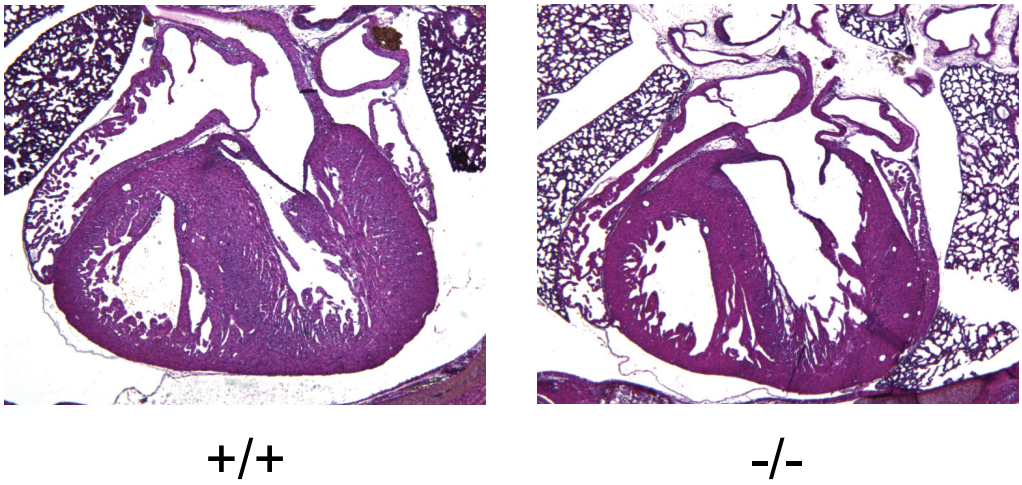

Fig. S3. Heart histology of Erk3-deficient mice. Rib cages of newborn Erk3$^{+/+}$ and Erk3$^{-/-}$ mice were fixed in formalin, embedded in paraffin and sectioned in the transversal plane. Heart sections were stained with hematoxylin and eosin.

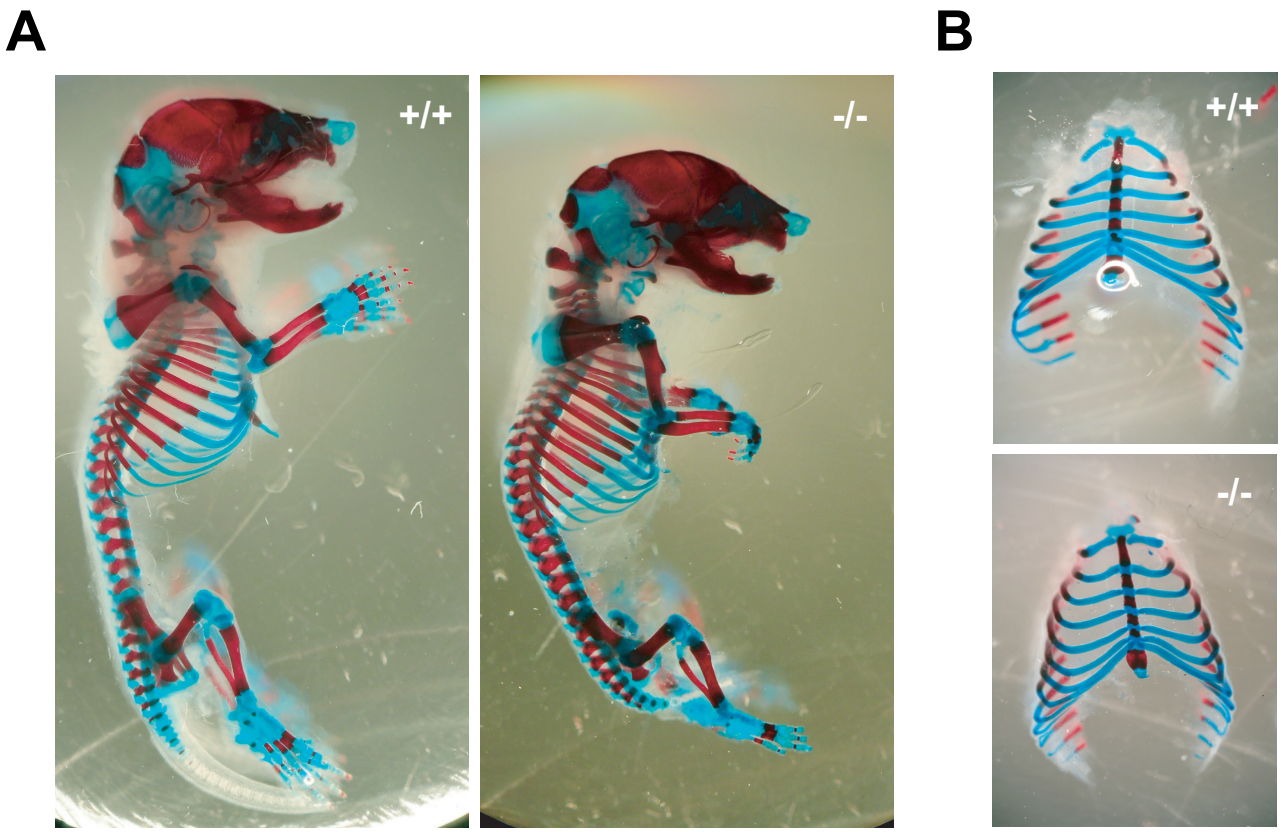

Fig. S4. Skeletal preparations of Erk3-deficient mice. Newborn Erk3$^{+/+}$ and Erk3$^{-/-}$ mice were double stained with Alizarin red (bone) and Alcian blue (cartilage). (*A*) Lateral view of whole skeletal preparation. Note the more pronounced curvature of the spine in Erk3$^{-/-}$ mouse. (*B*) Ventral view of the rib cage.

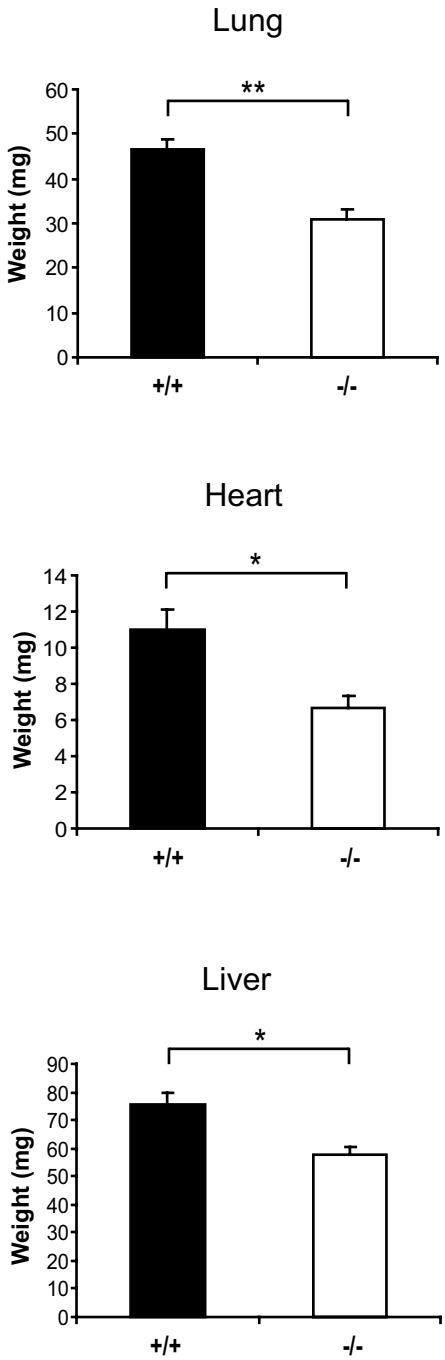

Fig. S5. Organ weight of Erk3-deficient embryos. Weight of the lung, heart, and liver in wild type (filled bars) and Erk3$^{-/-}$ (open bars) E18.5 embryos ($n = 8–13$). *, $P < 0.01$, **, $P < 0.001$.

Other Supporting Information Files

Table S1
Table S2