

# MEK kinase 1 activity is required for definitive erythropoiesis in the mouse fetal liver

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Mitogen-activated protein kinase/extracellular signal to regulated kinase (MEK) kinase 1 (MEKK1) is a c-Jun N-terminal kinase (JNK) activating kinase known to be implicated in proinflammatory responses and cell motility. Using mice deficient for MEKK1 kinase activity (*Mekk1*<sup> $\Delta KD$ </sup>) we show a role for MEKK1 in definitive mouse erythropoiesis. Although *Mekk1*<sup> $\Delta KD$ </sup> mice are alive and fertile on a 129 × C57/BL6 background, the frequency of *Mekk1*<sup> $\Delta KD$ </sup> embryos that develop past embryonic day (E) 14.5 is dramatically reduced when backcrossed into the C57/BL6 background. At E13.5, *Mekk1*<sup> $\Delta KD$ </sup> embryos have normal morphology but are anemic due to failure of definitive erythropoiesis. When *Mekk1*<sup> $\Delta KD$ </sup> fetal liver cells were transferred to lethally irradiated wild-type hosts, mature red blood cells were generated from the mutant cells, suggesting that MEKK1 func-

tions in a non-cell-autonomous manner. Based on immunohistochemical and hemoglobin chain transcription analysis, we propose that the failure of definitive erythropoiesis is due to a deficiency in enucleation activity caused by insufficient macrophage-mediated nuclear DNA destruction. (Blood. 2005;106:3396-3404)

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# Introduction

Production of red blood cells (erythropoiesis) is an essential step in vertebrate embryogenesis making it possible to advance from diffusion-restricted to circulation-assisted growth. In the mouse embryo, primitive erythropoiesis is initiated in the yolk sac around embryonic day (E) 7.5. Yolk sac-derived erythrocytes are large, nucleated cells that do not complete the final stages of normal adult erythropoiesis.<sup>1</sup> Around E9.5 erythropoiesis is initiated in the fetal liver<sup>2,3</sup>; concurrently, the yolk sac gradually becomes less important for erythropoiesis and around E14.5 90% of the erythrocytes present in circulation are of fetal liver origin.<sup>4,5</sup> The generation of mature erythrocytes involves the commitment of pluripotent hematopoietic stem cells progressing through erythroid blast-forming unit (BFU-E) and erythroid colony-forming unit (CFU-E) progenitor-cell stages, proerythroblast and several erythroblast stages, ultimately terminating into mature enucleated erythrocytes. Moreover, the development of fetal liver erythropoiesis is considered to proceed through 2 distinct phases: the first phase (E11-13), in which erythroid maturation proceeds in the absence of macrophages, and the second phase, involving the establishment of erythroblastic islands consisting of a central macrophage surrounded by erythroblasts.6 The central macrophages of the erythroblastic islands have been suggested to induce several key biologic processes in the erythroid precursors including proliferation and differentiation, prevention of apoptosis, and promotion of erythroblast enucleation.<sup>7</sup> Furthermore, recent studies suggest that failure of definitive erythropoiesis in deoxyribonuclease (DNase) II– deficient mice is due to impaired erythroblast enucleation, caused by inability of the central macrophages to digest nuclear DNA expelled from erythroblasts.<sup>8,9</sup>

The conserved mitogen activated protein kinase (MAPK) family members c-Jun N-terminal kinase (JNK) and p38 have been implicated in stress and proinflammatory signal transduction and recently in erythropoiesis.<sup>10-12</sup> A number of cytokines are involved in the erythropoiesis, including erythropoietin and stem-cell factor (SCF),<sup>13,14</sup> and it has been shown that erythropoietin induces sustained JNK activity and supports proliferation of erythropoietindependent cell lines<sup>12</sup>; additionally, p38 seems to be required for posttranscriptional regulation of erythropoietin expression.<sup>10</sup> Homozygous deletion of either JNK1 or JNK2 in mice has no apparent effect on hematopoiesis,<sup>15,16</sup> which might be explained by redundancy between the JNK isoforms. In contrast, the Jnk1-/-Jnk2-/embryos die early in embryonic development (E11.5-12.5) due to dysregulation of apoptosis in brain development, making the contribution of the JNK kinases in definitive erythropoiesis in vivo difficult to study further. We have investigated the role of the JNKactivating kinase mitogen-activated protein kinase/extracellular signal to regulated kinase (MEK) kinase 1 (MEKK1) in development. Mice deficient in MEKK1 kinase activity ( $Mekk1^{\Delta KD}$  mice)

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were generated and are alive and fertile on a C57/BL6  $\times$  129 background.<sup>17,18</sup> Unexpectedly, when backcrossing the *Mekk1*<sup>Δ*KD/+*</sup> heterozygotes into the C57/BL6 background, we observed a dramatic decrease in the frequency of live *Mekk1*<sup>Δ*KD*</sup> embryos that develop past E14.5. At E14.5 all mutant embryos studied, although morphologically normal, were anemic and showed defective definitive erythropoiesis with accumulation of nucleated late erythroblasts.

### Materials and methods

#### Mice

 $Mekkl^{\Delta KD}$ ,  $Jnkl^{-/-}$ , and  $Jnk2^{-/-}$  mice have been described previously.<sup>15,18,19</sup> The  $Mekkl^{\Delta KD}$ ,  $Jnk1^{-/-}$ , and  $Mekkl^{\Delta KD} Jnk2^{-/-}$  mice were obtained by crossing  $Mekkl^{\Delta KD/+}$  (C57BL/6 × 129/F1) with  $Jnk1^{-/-}$  (C57BL/6) or  $Jnk2^{-/-}$  (C57BL/6) mice.  $Mekkl^{\Delta KD/+}$  (C57BL/6 × 129/F1) were backcrossed to C57BL/6 for 8 generations. Mouse tail DNA was prepared from approximately 1-mm tail snips of embryos and 4-week-old progeny. Genotyping was performed by polymerase chain reaction (PCR) analysis of tail- and embryo-derived DNA.

#### **Tissue staining**

For endothelial stainings, E13.5 sections of acetone-fixed, paraffinembedded mouse embryos were stained with MECA-32 Rat  $IgG_{2a}$  (B&D Biosciences, San Jose, CA) against mouse panendothelial-cell antigen followed by biotin antirat antibody, and enhanced using the TSA system as described by the manufacturer (NEN, Boston, MA). For macrophage stainings, raw frozen sections of wild-type (wt) and *Mekk1*<sup>ΔKD</sup> embryos from E13.5 were stained with phycoerythrin (PE)–conjugated F4/80 (Caltag, Burlingame, CA) and afterward fixed with 4% paraformaldehyde.

Frozen sections of wt and  $Mekkl^{\Delta KD}$  mice from E13.5 were used for detection of apoptosis using the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) digoxigenin nick endlabeling (TUNEL) staining method. Briefly, sections were boiled in 0.1 M citrate buffer (pH 6.0) in a microwave oven for 2 minutes to permeabilize apoptotic cells. After cooling down, slides were incubated in 0.1 M Tris-HCl, 20% bovine serum albumin (BSA), 3% fetal porcine serum, for 30 minutes at room temperature and immersed in the TUNEL reaction mixture (Roche, Mannheim, Germany) for 1 hour at 37°C in a humidified chamber. Positive cells were visualized in a fluorescence microscope. Slides were viewed using an Axioskop 2 microscope equipped with Plan NEOFLUAR 10×/0.30 or 40×/0.75 objective lenses (Zeiss, Oberkochen, Germany). Images were captured with a ColorSnap digital camera (Photometrics, Tucson, AZ), and were processed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD) and Adobe Photoshop 7.0 software (Adobe, San Jose, CA).

#### Reconstitution of hematopoiesis in lethally irradiated mice

Fetal liver (FL) cells from wt, *Mekk1*<sup> $\Delta KD$ </sup>, *Jnk2*<sup>-/-</sup>, or *MEKK Jnk2*<sup>-/-</sup> E14.5 embryos were used to reconstitute lethally irradiated B6.SJL (CD45.1) female mice, exposed to 950 rad of  $\gamma$  irradiation. A total number of 1  $\times$  10<sup>6</sup> FL cells were injected into the tail vein of the host. Reconstitution of hematopoiesis (red blood cell [RBC] quantity, hematocrit value, hemoglobin concentration, mean corpuscular volume of erythrocytes, white blood cell [WBC] quantity, neutrophil, lymphocyte, monocyte, macrophage, eosinophil, and basophil percentages of total WBCs as well as platelet quantity) was examined with an automatic cell analyzer (Sysmex SF-3000; Kobe, Japan) every 2 weeks after reconstitution.

#### Flow cytometry

Single-cell suspensions of wt,  $Mekk1^{\Delta KD}$ ,  $Jnk2^{-/-}$ , or  $Mekk1 Jnk2^{-/-}$  fetal livers were obtained at E13. For analysis of reconstituted mice, peripheral blood was collected from retro-orbital veins, and lysis of erythrocytes was

performed. Staining of cells for flow cytometry was performed in 0.5% BSA in phosphate-buffered saline (PBS) using the following monoclonal antibodies: fluorescein isothiocyanate (FITC)–conjugated anti-CD44 (IM7), anti-CD34 (RAM34) PE, anti–c-kit (CD117) FITC, anti-Ter119 PE, anti-CD45.2 FITC, anti-CD45.1 PE, anti–Gr-1 PE, anti-CD11b (Mac1) PerCP-Cy5.5, anti-CD24 (HSA) biotin, anti-CD4 PE, and anti-CD43 PE (all from B&D Biosciences). Streptavidin TriColor (TC), anti-CD8 TC, and anti-B220 TC were from Caltag. Cell-surface expression of the different markers was analyzed in a Becton Dickinson FACScan Calibur using CellQuest Pro (B&D Biosciences) software; a minimum of 20 000 cells were counted.

#### **RNA** analysis

Total RNA was isolated from livers of wt and  $Mekk1^{\Delta KD}$ ,  $Jnk2^{-/-}$ , or  $Mekk1^{\Delta KD}$ ,  $Jnk2^{-/-}$  E13.5 embryos using the TRI REAGENT (Molecular Research Center, Cincinnati, OH) purification method as recommended by the manufacturer. For cDNA synthesis, 1 µg total RNA was converted to cDNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas) as recommended by the manufacturer. RNase protection assay was performed as previously described<sup>20</sup> using an mCK-4 Multi-Probe Template Set from BD Pharmingen (San Jose, CA). Band volume analysis was performed using a Typhoon 9410 variable mode imager and ImageMaster Totallab 2.0 software (Amersham Pharmacia Biotech, Piscataway, NJ). The concentrations of stem-cell factor (SCF), interleukin-3 (IL-3), IL-11, IL-7, GM-CSF, M-CSF, G-CSF, LIF, or IL-6 were normalized relative to those of glyceraldehyde-3-phosphate dehydrogenase (GADPH) and L32.

#### **Quantitative PCR**

Quantitative PCR-based measurement of RNA abundance was carried out using gene-specific double fluorescent-labeled probes and the Brilliant QPCR Master Mix (Stratagene, La Jolla, CA), which uses ROX (Rhodamine-X) passive reference dye to normalize for non-PCR-related fluorescence signal variation. 6-FAM (5-carboxyfluorescein) or in the case of GADPH, HEX (4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein) was the 5' fluorescent reporter, and Black Hole Quencher (BHQ-1) was added to the 3' end as a quencher. The following primer and probe sequences were used: Mgadph-571 forward primer (F), 5'-CAATGTGTCCGTCGTGGA-3'; mGADPH-654 reverse primer (R), 5'-GATGCCTGCTTCACCACC-3'; mGADPH-599 probe, 5'-CGCCTGGAGAAACCTGCCAAGTAT-3', mHga-105F, 5'-TAGCTTCCCCACCAACAA-3'; mHga-183R, 5'-CTTGC-CGTGACCCTTGAC-3'; mHga-133 probe, 5'-CTTCACATTTGATGTA-AGCCACGGC-3'; mHgβ-327F, 5'-TGCGATCGTGATTGTGCT-3'; mHgB-435R, 5'-CTTGTGAGCCAGGGCAGT-3'; mHgB-390 probe, 5'-CTTCCAGAAGGTGGTGGCTGGAGT-3'; mHgζ-157F, 5'-TTCTTAAC-CCCCCAAGCCC-3'; mHgζ-233R, 5'-TTCTTAACCCCCAAGCCC-3'; mHgζ-182 probe, 5'-TTAGAGCCCATGGCAAGAAAGTGC-3'; mEPO-61F, 5'-GGCCTCCCAGTCCTCTGT-3'; mEPO-168R, 5'-TGCACAAC-CCATCGTGAC-3'; mEPO-95 probe; 5'-TCTGCGACAGTCGAGTTCTG-GAGA-3'; mSCF-616F, 5'-AAGGCCCCTGAAGACTCG-3'; mSCF-701R, 5'-GCTCCAAAAGCAAAGCCA-3'; mSCF-636 probe, 5'-CCT-ACAATGGACAGCCATGGCATT-3'.

PCR was performed at 95°C for 10 minutes and then cycles at 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 30 seconds, on an Mx3000P real-time PCR system machine (Stratagene).

Results were analyzed as: relative gene transcription = 2(efficiency gene of interest / ct gene of interest – efficiency GADPH / ct GADPH), where ct indicates cycle threshold.

#### Macrophage phagocytosis assay

FL cells were isolated from E13.5  $Jnk2^{-/-}$ ,  $Mekk1^{\Delta K/+}$   $Jnk2^{-/-}$ , and  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$  embryos, and were resuspended in complete medium (RPMI plus 10% fetal bovine serum [FBS]) and allowed to attach to plastic (12-well dishes) for 90 minutes at 37°C in 5% CO<sub>2</sub>. Adherent macrophages (obtained by washing wells in RPMI to remove nonadherent cells) were offered an excess of latex beads (Sigma, St Louis, MO) and further incubated in complete medium for 3 hours. Macrophages were washed in

RPMI and stained with hematoxylin. The number of phagocytosed beads per cell were determined by phase-contrast microscopy.

#### **Reconstitution of FLM-erythroblast clusters**

Clusters composed of fetal liver macrophages (FLMs) and erythroblasts were isolated as described.<sup>21</sup> Briefly, FLs from wt or *Mekk1*<sup> $\Delta KD$ </sup> E13.5 embryos were placed in prewarmed 0.05% collagenase (Sigma) and 0.002% DNase (Sigma) in RPMI and digested for 1 hour at 37°C with gentle rotation. After tissue dissociation, cells were washed 3 times, suspended in 300 µL complete medium (RPMI plus 10% FBS) and allowed to attach to glass coverslips for 20 minutes at 37°C in 5% CO<sub>2</sub>. Cells were then flooded with medium and incubated for an additional 4 hours. Adherent macrophages with attached erythroid clusters were obtained by washing coverslips in RPMI to remove nonadherent cells ("native" clusters) before staining with F4/80-PE (Caltag) and biotin-labeled Ter119 antibody (Caltag) followed by visualization using the tyramide amplification system as recommended by the manufacturer, and streptavidine coupled to Alexa 488 (Molecular Probes, Eugene, OR).

### Results

# *Mekk1* $^{AKD}$ and *Mekk1* $^{AKD}$ *Jnk2* $^{-/-}$ embryos die in midgestation and are anemic

To identify at which developmental stage embryos deficient in MEKK1 kinase activity die, the phenotypes and genotypes of offspring from  $Mekk1^{\Delta KD/+}$  intercrosses (backcrossed into C57/BL6 for 8 generations) were examined between E8.5 and E18.5. Live  $Mekk1^{\Delta KD}$  embryos at Mendelian frequencies were detected up to midgestation (Table 1). Thereafter, the frequency was considerably lower than expected. To confirm that the effect of MEKK1 in development is mediated through JNK, we crossed the  $Mekk1^{\Delta KD}$  to JNK1-deficient  $(Jnk1^{-/-})$  and JNK2-deficient  $(Jnk2^{-/-})$  mice.

Although a small number of  $Mekk1^{\Delta KD}$  mice survived to adulthood, the double-deficient mice  $(Mekk1^{\Delta KD} Jnk2^{-/-}$  and  $Mekk1^{\Delta KD} Jnk1^{-/-}$ ) all died during embryonic development (Table 1). The phenotype of  $Mekk1^{\Delta KD} Jnk1^{-/-}$  and  $Mekk1^{\Delta KD} Jnk2^{-/-}$  was identical to that of the  $Mekk1^{\Delta KD}$  embryos (Figure 1, data not shown). However, as the breeding efficiency of the  $Mekk1^{\Delta KD/+} Jnk1^{-/-}$  intercrosses was very low, only limited studies were performed on these mice. The majority of the work in this study was performed on  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD} Jnk2^{-/-}$  mice.



Figure 1. *Mekk1*<sup>Δ*kD*</sup> and *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> embryos are anemic and exhibit a striking increase in the number of nucleated erythrocytes. (A) E14.5 wt, *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> mouse embryos (Theiler stages 1, 2). Note the absence of color in major blood vessels, the general paleness, and the smaller liver of the *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> embryo. (B) Sections through major blood vessels of *wt* and *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> embryo. (B) Sections through major blood vessels of *wt* and *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> embryo. (B) Sections through major blood vessels of *wt* and *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> embryo. (B) Sections through major blood vessels of *wt* and *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> and *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> (E14.5) embryos stained with H&E. Note the almost complete absence of enucleated erythrocytes in the *Mekk1*<sup>Δ*kD*</sup> *and Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup>, and *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> (E14.5) embryos stained with H&E. Note marked decrease in the number of circulating erythrocytes in the *Mekk1*<sup>Δ*kD*</sup> *Mek*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> samples. Bar = 100 μm. (D) Subcutaneous panendothelium stained sections of E13.5 *wt*, *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup>, and *Mekk1*<sup>Δ*kD*</sup> *Jnk2*<sup>-/-</sup> embryos. Bar = 100 μm.

At E14.5, live  $Mekkl^{\Delta KD}$  and  $Mekkl^{\Delta KD}$   $Jnk2^{-/-}$  embryos, macroscopically, were paler than wt counterparts and their major blood vessels lacked color (Figure 1A). The FL was smaller, hypocellular, and liver necrosis as well as erythroplaki of the head

Table 1. Genotypes of live offspring from Mekk1 <sup>ΔKE</sup>	/+ intercrosses, <i>Mekk1<sup>∆KD/+</sup> Jr</i>	nk2 <sup>-/-</sup> intercrosses, and Mekk1 <sup>ΔKD/+</sup>	Jnk1 <sup>-/-</sup> intercrosses
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	MEKK1 genotype				JNK2 genotype			JNK1 genotype				
Stage	Total (no. of litters)	Wt (%)	<i>Mekk1<sup>∆KD/+</sup></i> (%)	<i>Mekk1<sup>∆KD</sup></i> (%)	Total (no. of litters)	Jnk2 <sup>-/-</sup> (%)	Jnk2 <sup>-/-</sup> Mekk1 <sup>ΔKD/+</sup> (%)	Jnk2 <sup>-/-</sup> Mekk1 <sup>∆KD</sup> (%)	Total (no. of litters)	Jnk1 <sup>-/-</sup> (%)	Jnk1 <sup>-/-</sup> Mekk1 <sup>4KD/+</sup> (%)	Jnk1 <sup>-/-</sup> Mekk1 <sup>∆KD</sup> (%)
E8.5	9 (1)	1 (11)	7 (78)	1 (11)	ND	ND	ND	ND	ND	ND	ND	ND
E10.5	9 (1)	3 (33)	4 (44)	2 (22)	6 (1)	1 (17)	3 (50)	2 (33)	ND	ND	ND	ND
E12.5	ND	ND	ND	ND	53 (6)	13 (25)	28 (53)	12 (23)	ND	ND	ND	ND
E13.5	68 (12)	18 (26)	29 (43)	21 (31)	78 (12)	23 (29)	40 (51)	15 (19)	11 (2)	1 (9)	7 (63)	3 (27)
E14.5	125 (19)	38 (30)	60 (48)	27 (22)	75 (12)	31 (41)	29 (39)	15 (20)	6 (2)	0 (0)	5 (83)	1 (17)
E15.5	70 (9)	17 (24)	47 (67)	6 (9)	41 (7)	7 (17)	28 (68)	6 (15)	8 (2)	3 (37)	5 (62)	0 (0)
E16.5	22 (3)	8 (36)	13 (59)	1 (5)	16 (3)	4 (25)	11 (69)	1 (6)	ND	ND	ND	ND
E17.5	18 (3)	5 (28)	12 (67)	1 (5)	36 (5)	10 (28)	26 (72)	0 (0)	ND	ND	ND	ND
E18.5	13 (2)	4 (31)	9 (69)	0 (0)	ND	ND	ND	ND	3 (1)	1 (33)	2 (66)	0 (0)
Postnatal	217 (42)	78 (36)	135 (62)	4 (2)	291 (57)	115 (40)	176 (60)	0 (0)	30 (11)	11 (37)	19 (63)	0 (0)

Surviving fetuses were defined as those with beating heart at the time of dissection. Unless noted otherwise, data are expressed as the number of embryos, with percentages within parenthesis.

ND indicates not determined.

and neck region was occasionally observed. In order to establish the cause of death, we performed various histologic and immunohistochemical analysis of E14.5 to E16.5  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD}$  $Jnk2^{-/-}$  embryos. Histologic examination of wt,  $Mekk1^{\Delta KD}$ , and  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$  fetuses revealed a marked increase in the number of nucleated erythrocytes in circulation. While blood vessels of wt and  $Jnk2^{-/-}$  embryos contained mostly enucleated erythrocytes; the erythrocytes found in blood vessels of  $Mekk1^{\Delta KD}$ and  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$  embryos were mostly nucleated and very few mature red blood cells were detected (Figure 1B; Table 2). The altered blood picture correlated with a decrease in the total amount of circulating erythrocytes in  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$ embryos (Figure 1C). These data show that  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD}$  $Jnk2^{-/-}$  fetuses are severely anemic just prior to their death.

The early lethality of the Jnk1-/-Jnk2-/- double-knockout embryos makes the total contribution of JNK in hematopoiesis difficult to study in vivo. Additionally, redundancy between the JNK isoforms may compensate for the loss of one gene or the other. Nevertheless, in order to investigate whether the relative contribution of JNK1 or JNK2 became more apparent during conditions of anemia, we examined reconstitution of hematopoiesis in Jnk1<sup>-/-</sup> and  $Jnk2^{-/-}$  mice 3 days after induction of severe anemia. However, Sysmex Counter Analysis revealed no differences compared with wt controls in any of the parameters investigated, including RBC quantity, hematocrit value, hemoglobin concentration, and mean corpuscular volume of erythrocytes (Supplemental Figure S1A-D, available at the Blood website; click on the Supplemental Figures link at the top of the online article). Similarly, the WBC quantity, percentages of neutrophils, basophils, eosinophils, monocytes, and lymphocytes were indistinguishable between  $Jnk1^{-/-}$ ,  $Jnk2^{-/-}$ , and wt mice (data not shown). These data suggest that redundancy between the JNK isoforms may compensate for the loss of either JNK1 or JNK2 also during hematopoiesis.

p38-deficient mice are anemic and succumb in 2 separate waves of embryonic lethality.<sup>10,22-24</sup> A fraction of the embryos develop past E16.5 where they die due to failed erythropoiesis.<sup>10</sup> However, the first critical time point occurs around E12.5. Two independent studies<sup>22,23</sup> have suggested that the early lethality is due to placental insufficiencies. Both studies noted an abnormal morphology with reduction in the labyrinthine layer and lack of intermingling embryonic and maternal blood vessels consistent with a lack of normal vascularization. We found no abnormalities in the morphology of placentas from  $Mekk1^{\Delta KD}$  or  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$  fetuses on E13.5. Gross morphology as well as closer examination revealed normal layer organization with fetal blood vessels penetrating into the labyrinthine and spongiotrophoblast layers (Supplemental Figure S2A-B). Neither could we observe any differences in endothelial development between wt,  $Mekk1^{\Delta KD}$ , and  $Mekk1^{\Delta KD} Jnk2^{-/-}$  fetuses as judged by immunohistochemical staining (Figure 1D).

#### Defective definitive erythropoiesis in *Mekk1*<sup>ΔKD</sup> embryos

We examined FL hematopoiesis in E13.5 wt,  $Mekk1^{\Delta KD}$ , and  $Mekk1^{\Delta KD}$  Jnk2<sup>-/-</sup> embryos by flow cytometry using markers that are characteristic of different hematopoietic cell types and developmental stages, including CD34 (detecting hematopoietic progenitors), CD44 (detecting all hematopoietic cells), c-kit (hematopoietic stem cells), and Ter119 (erythroid cells).<sup>25-28</sup>

Analysis of FL single-cell suspensions revealed an increase in the relative fraction of CD34<sup>low</sup>c-kit<sup>pos</sup> cells (representing a population of hematopoietic stem cells capable of long-term reconstitution)<sup>29</sup> in *Mekk1*<sup> $\Delta KD$ </sup> as well as *Mekk1*<sup> $\Delta KD$ </sup> *Jnk2<sup>-/-</sup>* fetal livers as compared with wt or *Jnk2<sup>-/-</sup>* FLs (Figure 2A). Fetal livers of *Mekk1*<sup> $\Delta KD$ </sup> and *Mekk1*<sup> $\Delta KD$ </sup> *Jnk2<sup>-/-</sup>* embryos also exhibited a modest increase in the relative abundance of CD34<sup>pos</sup>c-kit<sup>neg</sup> cells (Figure 2A).

The relative overproduction of hematopoietic stem cells and progenitors in *Mekk1*<sup> $\Delta KD$ </sup> and *Mekk1*<sup> $\Delta KD$ </sup> *Jnk2*<sup>-/-</sup> fetal livers could be a compensatory response to anemia. Indeed,  $MekkI^{\Delta KD}$  and  $Mekk1^{\Delta KD}$  Jnk2<sup>-/-</sup> fetal livers showed a substantial increase in Ter119pos CD44neg cells (Figure 2A). Ter119pos cells are committed erythropoietic precursors beyond the CFU-E stage<sup>25,27</sup> and CD44 expression is down-regulated during the final steps of erythroidcell differentiation.<sup>26</sup> These data support our histologic observations (Figure 1B) and suggest that the larger population of Ter119pos CD44<sup>neg</sup> cells observed in Mekk1<sup> $\Delta KD$ </sup> and Mekk1<sup> $\Delta KD$ </sup> Jnk2<sup>-/-</sup> as compared with control FLs represent an increase of cells very late in erythropoietic development. These results were confirmed using a recently developed flow cytometric assay<sup>30</sup> that distinguishes various stages of erythroid-cell differentiation based on doublestaining FL cells with anti-CD71 and anti-Ter119. Double staining of E13.5 FL cells from  $Mekk1^{\Delta KD}$  embryos revealed a substantial increase in the percentage of chromatophilic/orthocromatophilic erythroblasts as compared with wt littermate embryos (Supplemental Figure S3).

Further support for a defect in erythropoiesis at a stage beyond hemoglobin synthesis in *Mekk1*<sup> $\Delta KD$ </sup> and *Mekk1*<sup> $\Delta KD$ </sup> *Jnk2*<sup>-/-</sup> embryos was provided by quantitative (Q) PCR analysis of *globin* mRNA expression. The levels of  $\zeta$ -*globin* mRNA, which is expressed exclusively during primitive erythropoiesis,<sup>31</sup> were equal in wt, *Mekk1*<sup> $\Delta KD$ </sup>, and *Mekk1*<sup> $\Delta KD$ </sup> *Jnk2*<sup>-/-</sup> fetal livers (Figure 2B),

Table 2. Increased amount of orthochromatic normoblasts in <i>Mekk1</i> $^{\Delta KD}$ and <i>Jnk2</i> $^{-/-}$ <i>MEKK1</i> $^{\Delta KD}$ fetuse
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Stage	Genotype									
	Wt*		Mekk1 <sup>ΔKD</sup>	Jnk2 <sup>−/−</sup> Mekk1 <sup>∆KD</sup>						
	Orthochromatic normoblasts in circulation, %	N	Orthochromatic normoblasts in circulation, %	N	Orthochromatic normoblasts in circulation, %	N				
E11.5	100 ± 0	5	ND	ND	ND	ND				
E12.5	83 ± 4	6	ND	ND	ND	ND				
E13.5	44 ± 14	8	64	1	ND	ND				
E14.5	23 ± 8	7	72 ± 15†	6	83 ± 10†	7				
E15.5	2 ± 3	6	80	1	50 ± 15†	6				
E16.5	$0 \pm 0$	7	74	1	ND	ND				

Plus/minus values represent standard deviation. Values that are given without standard deviation are for cases in which only one mouse was analyzed. ND indicates not determined.

\*Jnk2<sup>-/-</sup> and Mekk1 $^{\Delta KD/+}$  were identical to wt.

 $\pm P$  < .05 compared with wt.



Figure 2. Definitive erythropoiesis is defective in Mekk1<sup>ΔKD</sup> and Mekk1<sup>ΔKD</sup> Jnk2<sup>-/-</sup> embryos. Flow cytometry of FL hematopoietic cells from E13.5 wt, Mekk1<sup>ΔKD</sup>, Jnk2<sup>-1</sup> and  $Mekk_{1\Delta KD} Jnk_{2^{-/-}}$  embryos and relative expression of globin genes in E13.5 FL of wt, Mekk11AKD, Jnk2-/-, and Mekk1<sup>ΔKD</sup> Jnk2<sup>-/-</sup> embryos. (A) Expression of different cellsurface markers is depicted. Cell suspensions from E13.5 FLs were labeled with anti-c-kit, which marks hematopoietic stem cells; anti-CD44, which marks all hematopoietic cells; anti-Ter119, to identify differentiated erythroid cells; or anti-CD34 to mark hematopoietic progenitors. The dot plots represent the log fluorescence intensities of live cells. (B) Relative expression of globin chains was measured by real-time PCR. The  $\zeta$  globin chain is found only in yolk sac–derived erythroblasts. The  $\alpha$  globin chain is found in both yolk sac-derived and FL-derived erythroblasts. The Bmajor alobin chain is found only in FL-derived ervthroblasts and is continuously expressed until just prior to enucleation. GADPH mRNA levels were used for normalization in all cases. Percentage of cells in each quadrant is indicated. Data represent the average ± standard deviation (SD) from 3 to 5 animals per group.

showing that the nucleated erythroblasts found in the circulation of  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$  embryos are not yolk sac derived. The levels of  $\alpha$ -globin, expressed both during primitive and definitive erythropoiesis and the expression of  $\beta^{major}$ -globin transcripts, which first appear in the FL during definitive erythropoiesis, were also similar in wt,  $Mekk1^{\Delta KD}$ , and  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$  FLs (Figure 2B). As the  $\beta^{major}$ -globin expression maximum is not reached until the polychromatic erythroblast stage,<sup>32-34</sup> the defect in the erythropoietic development must occur beyond this stage. We also analyzed hemoglobin chain expression at the protein level by tricine gel analysis<sup>8</sup> and no abnormalities were observed (data not shown).

These results were further confirmed by measuring the ability of cells, derived from E14.5 wt and  $Mekkl^{\Delta KD}$  FLs, to form immature BFU-Es as well as the more mature CFU-Es. These colony types reflect the presence of committed erythroid progenitors.<sup>35</sup> As expected, we observed no significant difference in the frequency of immature BFU-Es, mature BFU-Es, and CFU-Es formed from wt and  $Mekkl^{\Delta KD}$  fetal liver cells (Supplemental Figure S4A-C). We also examined the ability of  $Mekkl^{\Delta KD}$  FLs in leukocyte development and found no defects in granulocyte macrophage–CFU (CFU-GM) or granulocyte-erythrocyte-macrophage-megakaryocyte–CFU (CFU-GEMM) development (Supplemental Figure S4D-E). Collectively, these results show that primitive blood-cell development proceeds normally in  $Mekkl^{\Delta KD}$  and  $Mekkl^{\Delta KD}$  Jnk2<sup>-/-</sup> embryos but definitive erythropoiesis is severely defective at the orthochromatic erythroblast stage.

# Hematopoietic growth factors and cytokines are produced in normal amounts in $Mekk1^{\Delta KD}$ embryos

Numerous studies have implicated the MAP kinases in the production of various cytokines as well as in the cellular response to these cytokines. We therefore explored the possibility that MEKK1 may be required for optimal expression of erythropoietin, SCF, or other hematopoietic growth factors. RNA was extracted from FLs of morphologically normal and viable E13.5 wt, *Mekk1*<sup>ΔKD</sup>, *Jnk2*<sup>-/-</sup>, and *Mekk1*<sup>ΔKD</sup> *Jnk2*<sup>-/-</sup> embryos. The RNA levels of erythropoietin and SCF were first examined by Q-PCR analysis and the relative amount of IL-3, IL-11, IL-7, GM-CFS, M-CSF, G-CSF, LIF, IL-6, and SCF RNA transcripts was further examined by RPA analysis. However, no difference in the relative amounts of RNA was detected between wt,  $Mekk1^{\Delta KD}$ , and  $Mekk1^{\Delta KD} Jnk2^{-/-}$  samples (Figure 3A-B). To confirm that the RNA expression levels were reflected at the protein level, we performed Western blot analysis of FL protein extractions for SCF and erythropoietin, as well as immunohistochemistry for SCF. No differences were found (data not shown).

# $Mekk1^{\Delta KD}$ hematopoietic stem cells reconstitute lethally irradiated hosts

Our data suggest that the major aberration in  $Mekkl^{\Delta KD}$  and *Mekk1* $^{\Delta KD}$  Jnk2<sup>-/-</sup> embryos, which lead to a defect in definitive erythropoiesis, is insufficient enucleation. To examine whether the enucleation deficiency was intrinsic to the  $Mekkl^{\Delta KD}$  and  $Mekkl^{\Delta KD}$ Jnk2<sup>-/-</sup> maturing blood cells or extrinsic, we tested the ability of  $Mekkl^{\Delta KD}$  and  $Mekkl^{\Delta KD} Jnk2^{-/-}$  fetal liver hematopoietic stem cells (HSCs) to reconstitute hematopoiesis in lethally irradiated hosts. Adult B6.SJL mice, which express the CD45.1 antigen, were lethally irradiated and injected with single-cell suspensions of E14.5  $Jnk2^{-/-}$  or  $Mekk1^{\Delta KD} Jnk2^{-/-}$  FL cells, which express the CD45.2 antigen. While mock-injected lethally irradiated mice did not survive beyond 2 weeks, mice reconstituted with either  $Jnk2^{-/-}$ or *Mekk1* $^{\Delta KD}$  Jnk2<sup>-/-</sup> FL cells survived until the end of the study (4 months). Every 2 weeks after reconstitution, peripheral blood was analyzed for RBC quantity, hematocrit value, hemoglobin concentration, mean corpuscular volume of erythrocytes, WBC quantity, and percentages of neutrophils, lymphocytes, monocytes, macrophages, eosinophils, and basophils as well as platelet quantity by Sysmex Counter Analysis (Figure 4A and Supplemental Figure S5). The mean corpuscular volume of erythrocytes correlates with their maturation state; orthochromatic erythroblasts are much larger than mature erythrocytes and can therefore be envisioned by this analysis. Furthermore, blood smears were made



**Figure 3. Cytokine production in** *Mekk1*<sup>ΔKD</sup> *Jnk2*<sup>-/-</sup> **fetal livers are unaltered.** (A) Quantitative (Q) PCR analysis of erythropoetin and *SCF* cDNA from E13.5 wt, *Mekk1*<sup>ΔKD</sup>, *Jnk2*<sup>-/-</sup>, and *Mekk1*<sup>ΔKD</sup> *Jnk2*<sup>-/-</sup> FLs (n = 5).  $\Box$  indicates wt;  $\Box$ , *MEKK1*<sup>ΔKD</sup> *JNK2*<sup>-/-</sup>. Data represent average ± SD. (B) Representative samples from RNase protection analysis of *SCF*, EPO, *IL-3*, *IL-11*, *IL-7*, *GM*-*CSF*, *M-CSF*, *G-CSF*, *LIF*, and *IL-6* mRNA expression in E13.5 *Jnk2*<sup>-/-</sup> and *Mekk1*<sup>ΔKD</sup> *Jnk2*<sup>-/-</sup> FLs. GADPH and L32 were measured as controls. GM-CSF indicates granulocyte-macrophage colony-stimulating factor; GADPH, glyceraldehyde-3 phosphate dehydrogenase; and LIF, leukemia inhibitory factor.

from the peripheral blood and analyzed for the presence of nucleated erythrocytes; all samples from  $Jnk2^{-/-}$  and  $Mekkl^{\Delta KD}$   $Jnk2^{-/-}$  reconstituted animals contained exclusively mature erythrocytes (data not shown).

Additionally, cell-surface markers characteristic of donor-

derived erythroid, myeloid, and lymphoid cells were analyzed by flow cytometry. The reconstitution capacity of  $Mekkl^{\Delta KD} Jnk2^{-/-}$ FL cells was identical to that of  $Jnk2^{-/-}$  cells, and in all cases remaining host-derived cells accounted for less than 5% of total CD45<sup>+</sup> cells. In all cases, complete reconstitution of erythroid, myeloid (granulocytes, monocytes), and lymphoid (B and T) cells was observed (Figure 4B). Additionally, fluorescence-activated cell sorting (FACS) analysis of bone marrow cells from the reconstituted animals revealed no difference in percentages of donorderived bone marrow macrophages (Jnk2<sup>-/-</sup>,  $13.3\% \pm 1.2\%$ ;  $Mekkl^{\Delta KD}$   $Jnk2^{-/-}$ , 14.2%  $\pm$  1.6%; n = 4). Nor did hematoxylin and eosin (H & E) stains on sections from the bone marrow of the reconstituted animals show any abnormalities (Supplemental Figure S6). Thus, the erythropoietic deficiency of  $Mekkl^{\Delta KD} Jnk2^{-/-}$ mice is not cell autonomous and can be rescued by host-derived factor(s). We also tested the ability of  $Mekkl^{\Delta KD}$  FL cells to reconstitute lethally irradiated hosts with identical results (data not shown).

#### Reduced number of FL macrophages in *Mekk1*<sup>ΔKD</sup> embryos

To examine the cause for the impaired enucleation activity in  $Mekkl^{\Delta KD}$  and  $Mekkl^{\Delta KD}$   $Jnk2^{-/-}$  embryos, we analyzed FLs for the presence and function of macrophages. Immunohistochemical staining, using the macrophage-specific marker F4/80, indicated reduced numbers of FLMs in  $Mekkl^{\Delta KD}$  and  $Mekkl^{\Delta KD}$   $Jnk2^{-/-}$ embryos compared with wt or  $Jnk2^{-/-}$  mice (Figure 5B). We therefore analyzed E13.5 FL-cell suspensions by FACS for the presence of F4/80-positive cells. The percentage of FLMs in  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$  embryos was significantly reduced (3% compared with 8%; n = 5) in wt or  $Jnk2^{-/-}$  mice (Figure 5A). To examine whether MEKK1 is required for the ability of FLMs to bind erythroblasts, we used a recently described macrophage-erythroblast coculture assay.21 E13.5 FLs were partially digested with collagenase and FLMs were selectively allowed to bind to a substrate for a short-term incubation. The cultures were subsequently stained and analyzed by fluorescence microscopy for the presence of clusters composed of F4/80positive FLMs and Ter119-positive erythroblasts. Although substantially lower numbers of macrophages were present in the  $Mekkl^{\Delta KD}$ FL cultures, the macrophages present were able to bind Ter119positive cells (Figure 5C). Next, we analyzed whether the phagocytic ability of the FLMs was impaired, using an in vitro



Figure 4. Reconstitution of hematopoiesis by adoptive transfer of  $Jnk2^{-/-}$  and  $Mekk1^{\Delta KD} Jnk2^{-/-}$  fetal liver cells. Peripheral blood was drawn every 2 weeks after adoptive transfer of FL cells from  $Jnk2^{-/-}$  or  $Mekk1^{\Delta KD} Jnk2^{-/-}$  E14.5 embryos into lethally irradiated hosts. (A) Samples were analyzed for red blood cell (RBC) quantity, mean corpuscular volume (MCV) of the red blood cells, and white blood cell (WBC) quantity. (B) Peripheral blood cells were drawn 12 weeks after adoptive transfer. Samples were analyzed for cell-surface expression of different lineage markers. The dot plots represent the log fluorescence intensities of live cells (i), or live cells falling into the CD45.2 gate (ii-iv). Monoclonal antibodies used were against Ter119 (erythroid cells), Gr-1 (granulocytes), Mac1 (monocytes), CD4 and CD8 (T lymphocytes), B220 (B lymphocytes), and CD43 (leukocytes). The percentages of cells in each guadrant are indicated.



Figure 5. Decreased number of fetal liver macrophages in *Mekk1*<sup>ΔKD</sup> embryos. (A) Fetal liver cell suspensions from wt and *Mekk1*<sup>ΔKD</sup> E13.5 embryos were stained with macrophage-specific anti-F4/80 antibodies and analyzed by flow cytometry. The dot plots represent the log fluorescence intensities of live cells. Numbers indicate the percentage of F4/80-positive cells. (B) Immunohistochemical staining of E13.5 fetal liver sections from wt and *Mekk1*<sup>ΔKD</sup> embryos with PE-conjugated anti-F4/80. (C) Immunohistochemistry of "native" erythroblast-FLM clusters from wt and *Mekk1*<sup>ΔKD</sup> E13.5 embryos. Cell clusters were stained for F4/80 (red) and Ter119 (green). (D) E13.5 FLMs were isolated from *Jnk2*<sup>-/-</sup>, *Mekk1*<sup>ΔKD/+</sup> *Jnk2*<sup>-/-</sup>, and *Mekk1*<sup>ΔKD</sup> *Jnk2*<sup>-/-</sup> embryos and offered an excess of latex beads in an in vitro phagocytosis assay. Macrophages were counterstained with hematoxylin and the number of phagocytosed beads per cell was counted by phase-contrast microscopy. A minimum of 3 FLs was examined per genotype.

phagocytosis assay. The ability of  $Mekk1^{\Delta KD} Jnk2^{-/-}$  FLMs to engulf latex beads was not inferior to FLMs from wt embryos (Figure 5D). Moreover, transmission electron microscopy of E13.5 FL sections revealed the presence of erythoroblastic islands in  $Mekk1^{\Delta KD}$  FLs (Supplemental Figure S7). We next examined whether the clearance of apoptotic cells was impaired in  $Mekk1^{\Delta KD}$ and  $Mekk1^{\Delta KD} Jnk2^{-/-}$  FLs. Indeed, a substantial increase of TUNEL-positive cells was detected in  $Mekk1^{\Delta KD}$  FLs (Figure 6B, and in higher magnification D). These apoptotic foci did not colocalize with Ter119-positive cells (Supplemental Figure S8), suggesting that expelled nuclei from erythroblasts accumulate in  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD} Jnk2^{-/-}$  FLs. The accumulation of partly degraded DNA appeared specific to the liver, since we observed no increase of apoptotic foci in any other tissue (including brain, lung, heart, kidney, intestine, and extremities; data not shown).

A previous study has reported accumulation of phagolysosomes containing undigested nucleic material in FLMs from embryos lacking DNAse II.<sup>8</sup> However, transmission electron microscopy of FLMs from *Mekk1*<sup> $\Delta KD$ </sup> embryos revealed no abnormal structures (Supplemental Figure S7).

### Discussion

We have discovered a new physiologic function for the MAPK pathway in embryonic development. In the present study we provide evidence strongly suggesting that MEKK1-JNK signaling is required for degradation of nuclear DNA extruded from erythroid precursors during the late stages of definitive erythropoiesis in the fetal liver.

Several targeted gene approaches have been used to evaluate the biologic function of the JNK signaling pathway in mice. The first studies in which the JNK genes were individually disrupted produced mice that appeared morphologically normal, suggesting that the JNK genes were able to complement each other in most tissues. Animals deficient in both JNK1 and JNK3 or JNK2 and JNK3 survive normally, whereas Jnk1<sup>-/-</sup>Jnk2<sup>-/-</sup> mice die during embryonic development due to severe dysregulation of apoptosis in the brain.<sup>36</sup> The early lethality of the  $Jnk1^{-/-}$   $Jnk2^{-/-}$  doubleknockout embryos makes the total contribution of JNK in hematopoiesis difficult to estimate. However, the relative contribution of JNK1 or JNK2 may become more apparent during conditions of anemia in the  $Jnk1^{-/-}$  or  $Jnk2^{-/-}$  mice. We therefore examined reconstitution of hematopoiesis in Jnk1-/- and Jnk2-/- mice 3 days after challenging them with severe anemia. However, we observed no differences compared to wt controls in any of the parameters investigated, suggesting that redundancy between the JNK isoforms may compensate for the loss of either JNK1 or JNK2 also during hematopoiesis.

Mice deficient for the upstream JNK regulators MKK4 and MKK7 are also embryonic lethal. MKK4-deficient embryos, similarly to c-Jun–deficient animals,<sup>37,38</sup> suffer from severe anemia and die at E10.5 to E12.5 from abnormal liver development,<sup>39,40</sup> suggesting that MKK4 is required for c-Jun activation during embryonic development. The cause of death in *MKK7*<sup>-/-</sup> embryos remains inconclusive.<sup>41</sup> Disruption of activators further upstream of MKK4 and MKK7 within the MEKK family includes (in addition to MEKK1) MEKK2 and MEKK3. MEKK2-deficient mice develop normally and are fertile.<sup>42</sup> In contrast, mice lacking MEKK3 die at E11 due to failure of cardiovascular development.<sup>43</sup> Previous studies on MEKK1-deficient (*Mekk1*<sup>-/-</sup>) and MEKK1 kinase–deficient (*Mekk1*<sup>ΔKD</sup>) mice have suggested that these mice develop normally with the exception of a defect in eyelid closure, a



Figure 6. Defective degradation and accumulation of TUNEL-positive nucleic material in *Mekk1*<sup>ΔKD</sup> fetal livers. Histochemical analysis of FLs. Nucleic material in wt and *Mekk1*<sup>ΔKD</sup> FLs, as shown by TUNEL stain (green). Note the large amount of abnormal apoptotic foci in the *Mekk1*<sup>ΔKD</sup> fetal liver B, and in higher magnification D. A, B: bar = 75 µm; C, D: bar = 100 µm.

phenotype that is associated with control of cell migration.<sup>18,44</sup> However, the  $Mekk1^{-/-}$  or  $Mekk1^{\Delta KD}$  mice used in previous studies have, to the best of our knowledge, all been C57/BL6  $\times$  129 hybrids. We observed a skewing of the Mendelian frequencies of live pups born after intercrossing the Mekk $l^{\Delta KD/+}$  C57/BL/6 × 129 hybrids (Mekk $l^{+/+}$  = 29.2%).  $Mekk1^{\Delta KD/+} = 52.8\%$ ,  $Mekk1^{\Delta KD} = 19.9\%$ ; n = 232). This skewing was influenced by the genetic background, since fewer than 2%  $Mekkl^{\Delta KD}$  pups were born after backcrossing into the C57/BL6 background. Nevertheless, crossing the  $Mekkl^{\Delta KD/+}$  C57/BL/  $6 \times 129$  hybrids with Jnk1<sup>-/-</sup> or Jnk2<sup>-/-</sup> mice resulted in embryonic lethality of all  $Mekkl^{\Delta KD} Jnkl^{-/-}$  and  $Mekkl^{\Delta KD} Jnk2^{-/-}$ embryos (regardless of background), suggesting that an intact MEKK1-JNK signaling pathway is required for normal embryonic development. Mekk $1^{\Delta KD}$ , Mekk $1^{\Delta KD}$  Jnk $1^{-/-}$ , and Mekk $1^{\Delta KD}$  Jnk $2^{-/-}$ embryos have identical phenotypes, survive up to midgestation, and display normal morphology but are highly anemic. Interestingly, a similar phenotype has been observed in p38 $\alpha$ -deficient mice.<sup>10</sup> However, while the anemia in  $p38\alpha^{-/-}$  mice is attributed to defective erythropoietin gene expression, normal levels of erythropoietin at both mRNA and protein level were observed in FLs from  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$  embryos. In addition to erythropoietin, we also investigated the expression levels of other growth factors and cytokines suggested to be involved in hematopoietic development, including SCF, IL-3, IL-6, IL-7, IL-11, GM-CFS, M-CFS, G-CFS, and LIF. However, we observed no differences when compared with FLs from wt controls. The FLs of  $Mekkl^{\Delta KD}$ and *Mekk1* $^{\Delta KD}$  Jnk2<sup>-/-</sup> also contain BFU-Es and CFU-Es formed at normal frequencies, and can reconstitute erythropoiesis in lethally irradiated hosts. It is therefore clear that MEKK1 is not required for the production of, or the response to, cytokines and/or growth factors required for the expansion and differentiation of erythroid progenitors up to the stage where the orthochromatic erythroblasts lose their nuclei and become reticulocytes.

Interestingly, the phenotype of mice lacking DNaseII (a lysosomal DNase in macrophages) is almost identical to  $Mekl^{\Delta KD}$  and

*Mekk1* $^{\Delta KD}$  Jnk2<sup>-/-</sup> embryos. DNaseII<sup>-/-</sup> mice die before birth, suffer from severe anemia, and exhibit accumulation of erythroblasts that fail to undergo enucleation.<sup>8,9</sup> This anemic phenotype is a consequence of macrophages failing to aid in digestion of nuclear DNA expelled from erythroblasts during definitive erythropoiesis.<sup>8</sup> In our study, FLs from  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD} Jnk2^{-/-}$  embryos contain reduced levels of macrophages and TUNEL stains showed extensive accumulation of apoptotic bodies in the FLs of  $Mekkl^{\Delta KD}$ and  $Mekk1^{\Delta KD}$   $Jnk2^{-/-}$  mice. These apoptotic bodies did not colocalize with Ter119-positive erythroblasts but rather appeared free in the extra cellular space, suggesting that they represent nuclear DNA extruded from erythroid precursor cells. Since the phagocytic capacity of FLMs from  $Mekk1^{\Delta KD}$  and  $Mekk1^{\Delta KD}$  $Jnk2^{-/-}$  was found to be similar to wt fetal liver macrophages, our data indicate that the observed accumulation of nucleated erythroblasts is dependent quantitatively rather than qualitatively on FLMs. In conclusion, we have shown that a functional MEKK1-JNK signaling pathway is critically required for macrophageassisted, erythroblast nuclear enucleation during FL erythropoiesis. The role for macrophages in phagocytosis and degradation of erythroblast nuclei during erythropoiesis has been established.<sup>8,21</sup> Morover, FLMs are part of a delicate microenvironment where communication through soluble mediators (ie, cytokines), direct cell-to-cell contacts between stromal cells (including fibroblasts, endothelial cells, epithelial cells, and FLMs), and cell-matrix interactions are in crucial balance, ultimately supporting erythropoiesis. Whether or how the MEKK1-JNK signaling pathway is required for factors extrinsic to FLMs during development, differentiation, proliferation, and/or maturation remains to be investigated.

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