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Embryonic Lethality and Fetal Liver Apoptosis in Mice Lacking All Three Small Maf Proteins

Hiromi Yamazaki,[a] Fumiki Katsuoka,[b] Hozumi Motohashi,[c] James Douglas Engel,[d] and Masayuki Yamamoto[a]

Department of Medical Biochemistry,[a] Department of Bioscience for Drug Discovery,[b] and Center for Radioisotope Sciences,[c] Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan, and Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, Michigan, USA[d]

Embryogenesis is a period during which cells are exposed to dynamic changes of various intracellular and extracellular stresses. Oxidative stress response genes are regulated by heterodimers composed of Cap'n'Collar (CNC) and small Maf proteins (small Mafs) that bind to antioxidant response elements (ARE). Whereas CNC factors have been shown to contribute to the expression of ARE-dependent cytoprotective genes during embryogenesis, the specific contribution of small Maf proteins to such gene regulation remains to be fully examined. To delineate the small Maf function *in vivo*, in this study we examined mice lacking all three small Mafs (MafF, MafG, and MafK). The small *Maf* triple-knockout mice developed normally until embryonic day 9.5 (E9.5). Thereafter, however, the triple-knockout embryos showed severe growth retardation and liver hypoplasia, and the embryos died around E13.5. ARE-dependent cytoprotective genes were expressed normally in E10.5 triple-knockout embryos, but the expression was significantly reduced in the livers of E13.5 mutant embryos. Importantly, the embryonic lethality could be completely rescued by transgenic expression of exogenous MafG under *MafG* gene regulatory control. These results thus demonstrate that small Maf proteins are indispensable for embryonic development after E9.5, especially for liver development, but early embryonic development does not require small Mafs.

Oxidative stress causes damage to DNA, proteins, and lipids and causes or exacerbates various human diseases, including cancers (e.g., reviewed in reference 30). However, cells usually do not succumb to such stresses, as cells retain machinery that acts to minimize oxidative damage by inducing cytoprotective defense enzymes. Indeed, several transcription factor families have been identified as important regulators of cytoprotective genes related to the oxidative stress response and redox homeostasis (reviewed in reference 4). One such transcription factor family is the Cap'n'Collar (CNC) proteins (reviewed in reference 22), which exert their function by forming a heterodimer with small Maf family members (9). Three small Maf proteins (small Mafs), MafF, MafG, and MafK, have been identified in mammals (reviewed in reference 21), and an additional member of the family, MafT, has been identified in fish (37). The CNC-small Maf heterodimers bind to *cis*-acting motifs named the Maf recognition element (MARE) (13), NF-E2 binding motif (31), antioxidant response element (ARE) (32), or the electrophile response element (EpRE) (5). The latter two *cis*-acting motifs represent similar binding motifs that have been identified in the regulatory regions of numerous genes encoding antioxidant and xenobiotic-metabolizing enzymes (11). Regulation of gene expression through the ARE and EpRE motifs has been shown to be critical for the oxidative stress response, and the CNC-small Maf heterodimers are the transcription factors attributable to that regulation (10).

The CNC family is composed of four closely related transcription factors, p45 NF-E2, Nrf1, Nrf2, and Nrf3, as well as two related factors, Bach1 and Bach2. The *in vivo* functions of CNC proteins have been elucidated by gene targeting in the mouse. In *Nrf2* knockout mice, induction of a battery of antioxidant and xenobiotic-metabolizing enzyme genes are severely impaired (11). *Nrf1* knockout mice exhibit fetal liver hypoplasia coincident with diminished ARE-dependent cytoprotective gene expression (2, 3). Recently, mouse genetic approaches revealed that p45 NF-E2, which is known to be critical for platelet production, contributes to the activation of ARE-dependent antioxidant genes in megakaryocytes (25). Thus, the CNC family transcription factors play important and likely overlapping roles in the regulation of ARE-dependent genes *in vivo*.

The small Mafs have been shown to be critical for the CNC factor binding to specific *cis*-acting motifs (reviewed in reference 22). The NF-E2 binding motif and ARE/EpRE motif are all encompassed by either 13-bp or 14-bp MARE sequences, which are composed of a central core and flanking regions. The central core sequence is identical to either the tumor-promoter response element (TRE; 13-bp type) or cyclic AMP (cAMP) response element (CRE; 14-bp type), while the flanking sequence harbors a critical GC sequence required for site binding. Recent structural and biophysical analyses revealed that the small Mafs specifically recognize these GC sequences, whereas the CNC proteins bind to the core sequence (18, 40). The acquisition of the flanking GC binding by small Mafs has rendered the CNC-small Maf heterodimers to recognize a totally distinct set of target genes differing from those of the AP1 or CREB/ATF family members (18, 40). Target genes regulated by the CNC-small Maf heterodimers are important for hematopoiesis (p45 NF-E2-small Mafs) and stress response/cytoprotective gene expression (Nrf2/Nrf1/Bach1-small Mafs). How-

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Address correspondence to Masayuki Yamamoto, masiyamamoto@med.tohoku.ac.jp.
H. Yamazaki and F. Katsuoka contributed equally to this article.
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ever, any physiological contribution of the small Mafs to cytoprotective gene expression has not been elucidated *in vivo*.

In order to examine the roles small Mafs play *in vivo*, we and others have ablated each member of small Maf family proteins individually in mice (17, 28, 33). We found that *MafG* knockout mice showed mild thrombocytopenia and motor ataxia, while *MafF* and *MafK* knockout mice did not show apparent phenotypes. Since small Maf proteins are functionally redundant and expressed in an overlapping manner, we surmised that remaining small Maf protein expression compensated for the lost function of any individual. Supporting this notion, *MafG MafK* double-knockout mice display severe thrombocytopenia and neurological phenotypes and die before weaning (14, 16, 29).

To delineate the contribution of small Maf proteins in the absence of complementation by other family members, we generated small *Maf* triple-knockout mice, bearing mutations in all six alleles (i.e., $MafF^{-/-}$ $MafG^{-/-}$ $MafK^{-/-}$). During the course of the study, we found that small *Maf* triple-knockout mice were embryonic lethal (15). Therefore, we established mouse embryonic fibroblasts (MEFs) derived from the triple-knockout embryos and found that the induction of cytoprotective genes was severely compromised in the triple-knockout fibroblasts (15). Based on this result, we speculated that experiments attempting to decipher the phenotypes of small *Maf* triple-knockout mice might provide insight into the nature of CNC-small Maf heterodimer-mediated stress responses in the context of multilayered defense mechanisms. However, the exact phenotype of small *Maf* triple-knockout embryos and the contribution of small Mafs to gene expression *in vivo* remained to be clarified.

In this study, we analyzed in detail the phenotypes of small *Maf* triple-knockout mice, especially with regard to the regulation of ARE-dependent cytoprotective genes. We found that small *Maf* triple-knockout mice developed normally up to embryonic day 8.5 (E8.5), but found that the small Mafs are essential for embryonic development beyond E9.5. The small *Maf* triple-knockout mice exhibited growth retardation from E9.5 onward and died around E13.5. The mutant embryos showed severe hypoplasia in the fetal liver due to increased apoptosis of hepatocytes and erythrocytes. The expression of cytoprotective genes was decreased in the mutant livers, possibly leading to increased apoptosis. The embryonic lethality and liver hypoplasia observed in the small *Maf* triple-knockout mice could be completely rescued by transgenic expression of exogenous MafG. These observations thus demonstrate that small Maf-mediated gene expression is indispensable for the maintenance of fetal liver hepatocytes, but surprisingly, early embryogenesis to E8.5 appears to proceed normally without any contribution from the small Maf proteins.

MATERIALS AND METHODS

Generation of compound mutant mice. Germ line mutagenesis of the mouse *MafF*, *MafG*, and *MafK* genes was performed as described previously (28, 33). LoxP-flanked PGK-neo/TK or PGK-neo cassettes in all targeted alleles were removed by mating with *Ayu1-Cre* mice (26). All mice examined in this study were of a mixed background, with contributions from the 129Sv/J, C57BL/6J, and ICR strains. Genotypes were determined by PCR as described previously (28, 33). The body weight of each mouse was measured weekly. More than three independent animals of each genotype were first weighed on postnatal day 7 and then monitored to the 6th week.

Hematological analysis. Thirty to 50 microliters of peripheral blood was collected from individual 8- to 10-week-old mice. Hematopoietic indices were determined using a hemocytometer (Nihon Koden, Tokyo, Japan). Liver cells from E13.5 embryos were suspended in 2% fetal bovine serum/phosphate-buffered saline, and counted on a hemocytometer.

Quantitative RT-PCR. For quantitative reverse transcription-PCR (RT-PCR), total RNA was prepared from mouse embryo or fetal livers using an Isogen RNA extraction kit (Nippon Gene, Toyama, Japan) and following the manufacturer's protocol. cDNA was synthesized from total RNA by reverse transcriptase Superscript III (Invitrogen), and real-time PCR was performed using an ABI prism 7300 (Applied Biosystems), as previously described (28). The primer and probe sequences used for detecting *Nqo1*, glutathione *S*-transferase (GST) alpha 4 (*Gsta4*), *Gst* pi 1 (*Gstp1*), *Gst* pi 2 (*Gstp2*), thioredoxin reductase 1 (*Txnrd1*), *Gclc*, heme oxygenase 1 (*Hmox1*), and multidrug resistance proteins (*Mrp2*, *Mrp3*, *Mrp4*, and *Mrp5*) have been described previously (15, 27, 41). The rRNA primers and probes were purchased from Applied Biosystems.

Histological analysis. Whole embryos were fixed with 10% formalin (Mildform 10N; Wako, Tokyo) and embedded in paraffin using standard procedures. Sections (5 μm) were stained with hematoxylin-eosin. For single staining of cleaved caspase-3, anti-cleaved caspase-3 (Cell Signaling Technology) antibody was used. Diaminobenzidine was used as a chromogen, and hematoxylin was used for counterstaining. For double immunostaining, E13.5 livers were embedded in OCT compound (Sakura-Finetechnical, Tokyo, Japan). The frozen sections (7 μm) were fixed with 3% formalin. To detect erythroid cells and hepatocytes, anti-Ter119 (Becton Dickinson) and anti-delta-like homolog 1 (Dlk-1) (Medical and Biological Laboratories, Nagoya, Japan) antibodies were used, respectively. Visualization was performed using Alexa Fluor 488 for Ter119 and Dlk-1 and Alexa Fluor 594 for cleaved caspase-3 (Molecular Probes). Each first antibody was diluted to 1:200. Fluorescent images were observed using the LSM510 confocal imaging system (Carl Zeiss, Heidelberg, Germany).

Microarray analyses and data mining. Total RNA purified from mouse embryos was processed and hybridized to a whole-mouse genome microarray (4 × 44K; Agilent Technologies). Experimental procedures for Gene Chip were performed according to the manufacturer's protocol. Expression data were subjected to analysis with GeneSpring software (Silicon Genetics). Heat maps were generated by using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and JAVA Treeview (http://jtreeview.sourceforge.net/). The pathway analysis was performed using the Reactome pathway enrichment tool (http://www.reactome.org).

Plasmid construction for transgenic mouse analysis. To construct MGRD-LacZ and MGRD-MafG (where MGRD represents *MafG* regulatory domain), p2.9MafG-LacZ was first generated by subcloning a genomic fragment from the mouse *MafG* gene; SmaI-SmaI (kbp −2.9 to bp −8, with the translation start site designated +1) into the blunt-ended XhoI site of pSVβ (Clontech). The blunt-ended genomic fragment from the 3′ region of the *MafG* gene, NcoI (at kbp +1.1) to SphI (at kbp +4.8), was then inserted into the blunt-ended SalI site of p2.9MafG-LacZ to generate p2.9MafG-LacZ-3′. MGRD-LacZ was constructed by replacing the EcoRI-AflII fragment of p2.9MafG-LacZ-3′ with the 5.1-kbp fragment from the upstream region of the AflII site (at kbp −1.7) of the MafG genome. A *LacZ* cDNA was replaced with a histidine-tagged *MafG* cDNA (24) to generate MGRD-MafG.

Generation of transgenic mice. Transgene constructs were injected into C57BL/6 × BALB/c fertilized eggs. Transgenic mice were generated by standard methods (8). $MafF^{-/-}$ $MafG^{+/-}$ $MafK^{-/-}$ (*F0G1K0*) mice bearing the *MGRD-MafG* transgene (TG^{MafG} mice) were mated with *F0G1K0* mice to generate *F0G0K0*:TG^{MafG} mice. Genotypes were determined by PCR with a primer set for the *MGRD-LacZ* transgene (TG^{LacZ} construct) (5′-GCA TGA CTC GCC AGG AAC AG-3′ and 5′-GCA ACG AAA ATC ACG TTC TTG TTG G-3′) and a primer set for the TG^{MafG} construct (5′-AAG AAG GAG ATA TAC CAT GGG-3′ and 5′-GCA TTC TCC GAG GCC AGC TTC-3′).

Whole-mount LacZ staining. Mouse embryos were fixed at room temperature for 60 min in 1% formaldehyde, 0.2% glutaraldehyde, and 0.02% NP-40 in phosphate-buffered saline. 5-Bromo-4-chloro-3-

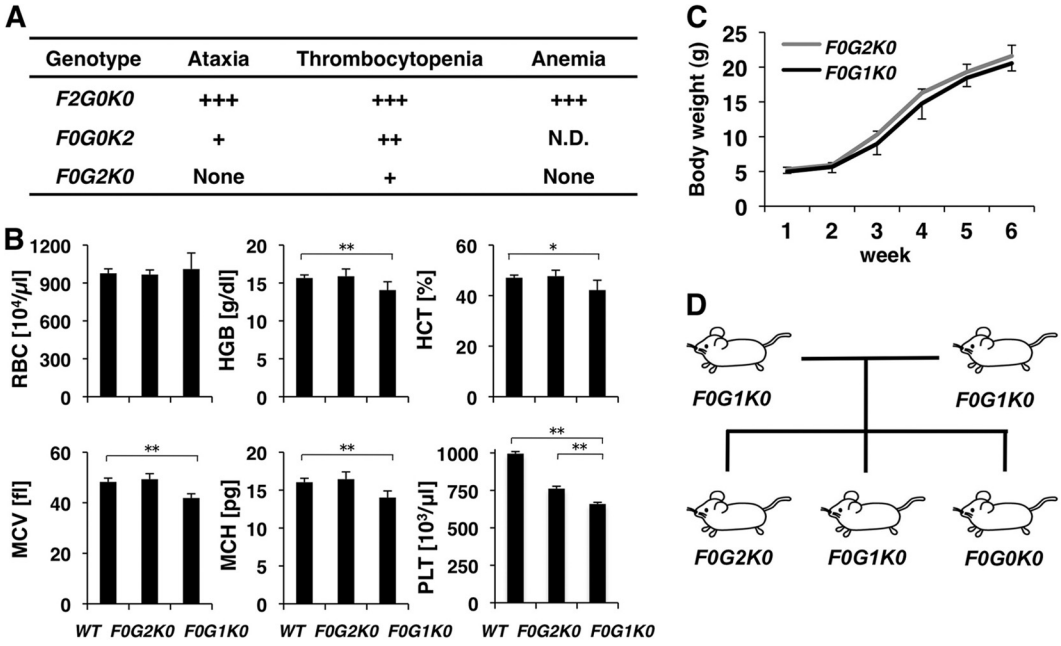

FIG 1 *F0G1K0* mice show no severe abnormality and are capable of producing small *Maf* triple-knockout mice. (A) Summary of phenotypes of small *Maf* double-knockout mice (14, 23, 28, 29, 33). Abnormalities were arbitrarily categorized into four groups: severe, +++; mild, ++; very mild, +; none. N.D., not determined. (B) Blood parameters in wild-type (WT), *F0G2K0*, and *F0G1K0* mice. Values are means ± standard deviations ($n = 7$ for wild-type mice, $n = 8$ for *F0G2K0* mice, $n = 6$ for *F0G1K0* mice). Student's *t* test was used to calculate statistical significance (*P*). *, $P < 0.05$; **, $P < 0.01$. (C) Body weight change for *F0G2K0* and *F0G1K0* mice. Values are means ± standard deviations ($n = 3$ to 4) (D) Mating strategy for producing small *Maf* triple-knockout mice. RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; PLT, platelet.

indolyl-β-D-galactosidase (X-Gal) staining was performed as previously described (33).

Microarray data accession number. The microarray data are available through the Gene Expression Omnibus database (accession no. GSE29558).

RESULTS

Generation of *MafF*$^{-/-}$ *MafG*$^{-/-}$ *MafK*$^{-/-}$ (*F0G0K0*) triple-knockout mice. In order to establish an efficient way to generate small *Maf* triple-knockout mice, we first asked whether animals harboring single-copy small *Maf* genes could become fertile adults. As summarized in Fig. 1A, it was found previously that, while *MafF*$^{+/+}$ *MafG*$^{-/-}$ *MafK*$^{-/-}$ (*F2G0K0*) and *MafF*$^{-/-}$ *MafG*$^{-/-}$ *MafK*$^{+/+}$ (*F0G0K2*) mice exhibit anemia, thrombocytopenia, and ataxia (14, 29, 33), *MafF*$^{-/-}$ *MafG*$^{+/+}$ *MafK*$^{-/-}$ (*F0G2K0*) mice are relatively healthy and fertile (23). Based on these observations, in this study we first examined the phenotypes of *F0G1K0* mice as candidates to generate the small *Maf* triple-knockout mice. We found that the *F0G1K0* mice exhibited mild hypochromic anemia and thrombocytopenia (Fig. 1B), perhaps due to impairment of the NF-E2 activity in the erythroid and megakaryocytic lineages (35). However, the *F0G1K0* mice did not exhibit any severe growth abnormality (Fig. 1C) or ataxia (data not shown). Most importantly, the *F0G1K0* mice were fertile and lived long enough to reproduce. These results demonstrate that one allele of the *MafG* gene is sufficient to sustain growth, survival, and fertility of mice. Hence, we decided to establish a breeding colony of *F0G1K0* mice to produce the small *Maf* triple-knockout mice efficiently (Fig. 1D).

Small *Maf* triple-knockout mice exhibit growth retardation and embryonic lethality. We intercrossed the *F0G1K0* mice and recovered 94 2-week-old mice. According to Mendelian law, the expected number of *F0G0K0* mice in the progeny of this intercross was 23 or more. However, no *F0G0K0* mice survived to this stage (Table 1). To characterize timing of the lethality, we conducted timed *F0G1K0* intercrosses and collected embryos at different stages of gestation (Table 2). We found *F0G0K0* embryos at the expected Mendelian frequency at E8.5. However, after E9.5 the frequency of *F0G0K0* embryos became lower than expected, and some of the *F0G0K0* embryos exhibited growth retardation (Fig. 2A, top panels). At E10.5, 33 out of 46 *F0G0K0* embryos showed growth retardation, whose severity varied greatly (data not shown). By E11.5, 13 out of 17 *F0G0K0* embryos exhibited severe growth retardation (Fig. 2A, middle panels). *F0G0K0* embryos are also pale compared with *F0G2K0* embryos at E13.5, implying that the embryos began to encounter inefficient hematopoiesis. At E13.5, 11/50 compound homozygous mutant embryos were recovered, and all of them showed severe growth retardation (Fig. 2A, bottom panels), indicating that most of the *F0G0K0* embryos died around E13.5. However, the timing of death was not precise, but rather was distributed widely during midgestation (Table 2).

During the course of embryo analyses, we observed one signif-

TABLE 1 Genotypes of viable offspring from *F0G1K0* mouse intercrosses

Parameter at 2 wk	No. of offspring of genotype:			Total
	F0G2K0	*F0G1K0*	*F0G0K0*	
Predicted	24	47	24	95
Observed	32	62	0	94

TABLE 2 Genotypes of viable offspring from *F0G1K0* mouse intercrosses

Stage and parameter	No. of offspring of genotype[a]:			Total
	F0G2K0	*F0G1K0*	*F0G0K0*	
E8.5				
Predicted	11	21	11	43
Observed	10	24	9	43
E9.5				
Predicted	19	39	19	77
Observed	24 (1)	42 (2)	11 (6)	77
E10.5				
Predicted	67	134	67	268
Observed	87 (6)	135 (2)	46 (33)	268
E11.5				
Predicted	25	50	25	100
Observed	22	61 (1)	17 (13)	100
E12.5				
Predicted	19	37	19	75
Observed	24 (2)	43 (1)	7 (5)	74
E13.5				
Predicted	50	99	50	199
Observed	73	115 (1)	11 (11)	199
E14.5				
Predicted	8	16	8	32
Observed	10	20	2 (2)	32
E15.5				
Predicted	12	24	12	48
Observed	17	30	1 (1)	48

[a] The numbers of embryos exhibiting growth retardation are shown in parentheses.

icant distinction in the mutants. The livers of *F0G0K0* embryos were very small compared to those of *F0G2K0* embryos (Fig. 2B), and the cell numbers in the livers were significantly reduced (Fig. 2C). All *F0G0K0* embryos exhibited diminished liver size (11 out of 11) (data not shown), while the sizes of other major organs, such as the heart or brain, were not severely affected (data not shown). These results demonstrate that the small Mafs are dispensable for early embryonic development up to E8.5, but they are essential for embryonic development beyond E13.5, and furthermore, they appear to be indispensable for fetal liver development.

Small Mafs contribute to the expression of cytoprotective genes in the fetal liver. It has been reported that *Nrf1* knockout mice exhibit growth retardation and severe hypoplasia in fetal livers, in which the expression of oxidative stress response genes was diminished (2, 3). Therefore, we surmised that the expression of ARE-dependent cytoprotective genes might be compromised in the *F0G0K0* embryo livers. We examined the expression of these cytoprotective genes and found that expression levels of many ARE-dependent genes (e.g., *Gclc*, *Txnrd1*, *Nqo1*, *Gstp2*, and several *Mrp* genes) were lower in the livers of *F0G0K0* embryos than in *F0G2K0* livers (Fig. 3).

We also examined the embryonic livers histologically. Hematoxylin-eosin staining of E13.5 *F0G2K0* embryo livers showed normal sinusoidal structure (Fig. 4A), which was severely damaged in the *F0G0K0* livers (Fig. 4B). Immunostaining of an apoptosis marker (cleaved caspase-3) clearly demonstrated the presence of apoptotic cells in *F0G0K0* fetal livers. The number of apoptotic cells was markedly higher in the *F0G0K0* embryo livers (Fig. 4D) than in *F0G2K0* livers (Fig. 4C). Double immunostaining with hematopoietic maker Ter119 or hepatic marker Dlk-1 (38) showed that apoptotic cells were observed in both the Ter119-positive cell fraction (Fig. 4E to H) and Dlk-1-positive cell fraction (Fig. 4I to L), suggesting that fetal liver hematopoiesis is impaired in *F0G0K0* embryos. Taken together, these results suggested that the basal expression of ARE-dependent genes in fetal livers is dependent on small Mafs, and this alteration could be one of possible causes of liver hypoplasia in *F0G0K0* embryos.

The expression of ARE-dependent cytoprotective genes is preserved in E10.5 *F0G0K0* embryos. The growth retardation is apparent in E10.5 *F0G0K0* embryos where livers are not yet formed (Table 2), supporting the contention that fetal liver hypoplasia may not account for the growth retardation in the mutants. To explore the cause of growth retardation observed in E10.5 *F0G0K0* embryos, we carried out a microarray analysis of gene expression at this stage. To eliminate differences simply caused by developmental delay, we compared the gene expression profile of E10.5 *F0G0K0* embryos with those of both E9.5 and E10.5 *F0G2K0* embryos. Our initial hypothesis was that the dysregulation of ARE-dependent cytoprotective gene expression might be a cause

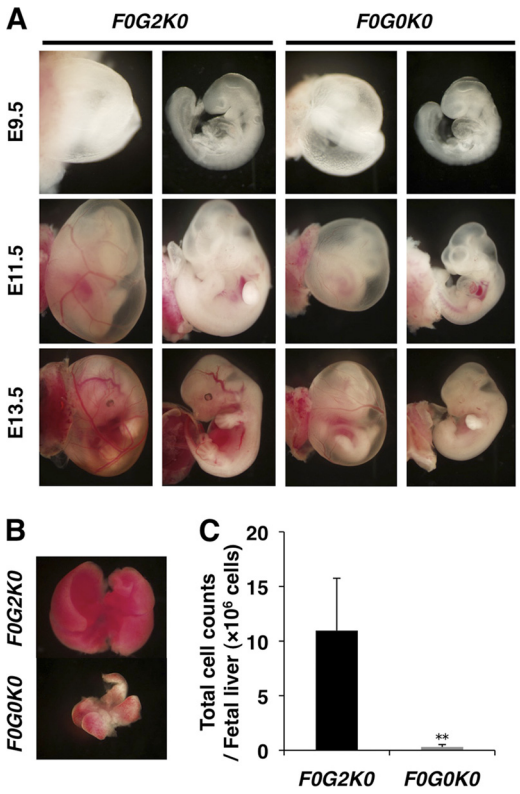

FIG 2 *F0G0K0* embryos show growth retardation and hypoplasia in fetal livers (A) Representative pictures of embryos with or without yolk sac at different developmental time points. The genotypes and gestational ages of the embryos are indicated. (B) Representative pictures of fetal livers for each genotype at E13.5. (C) Total cell numbers per fetal liver for each genotype at E13.5. Values are means ± standard deviations ($n = 3$ to 5). Student's *t* test was used to calculate statistical significance (*P*). **, $P < 0.01$.

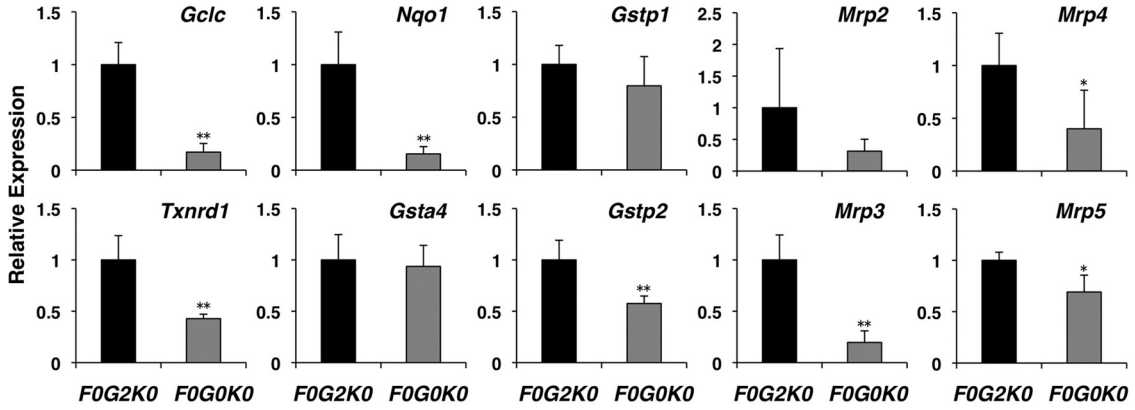

FIG 3 Expression profiles of antioxidant and xenobiotic-metabolizing enzyme genes in fetal livers at E13.5. Gene expression was examined by quantitative RT-PCR. The genotypes of the embryos are indicated. The expression level of each mRNA was normalized to the rRNA abundance. The average values of *F0G2K0* mice are set to 1. Values are means ± standard deviations ($n = 3$ to 5). Student's *t* test was used to calculate statistical significance (*P*). *, $P < 0.05$; **, $P < 0.01$.

of growth retardation. However, the expression levels of many ARE-dependent cytoprotective genes in *F0G0K0* embryos were comparable to those in *F0G2K0* embryos (Table 3).

To confirm the microarray data, we selected several ARE-dependent cytoprotective genes and performed quantitative RT-PCR analysis. In agreement with the microarray data, *Nqo1*, *Gsta4*, *Txnrd1*, and *Gclc* genes were normally expressed in *F0G0K0* embryo livers compared with those of *F0G2K0* embryos (Fig. 5). *Hmox1* gene expression was rather increased in *F0G0K0* embryos (Fig. 5), suggesting that small Mafs negatively regulate the *Hmox1* gene at this stage (15). These results thus demonstrate that the small Mafs are dispensable for the basal expression of these ARE-dependent cytoprotective genes at an early embryonic stage, and their diminished expression is not the cause of the growth retardation observed in the E10.5 embryos.

Exploration of small Maf-dependent genes in embryos. To explore potential causes of growth retardation or lethality in *F0G0K0* embryos, we first focused on genes whose expression were decreased more than 2.0-fold in E10.5 *F0G0K0* embryos in comparison with both E10.5 and E9.5 *F0G2K0* embryos. Pathway analysis revealed that genes related to hemostasis were strongly enriched in the downregulated genes (see Table S1 in the supplemental material). We also noticed that there are several genes

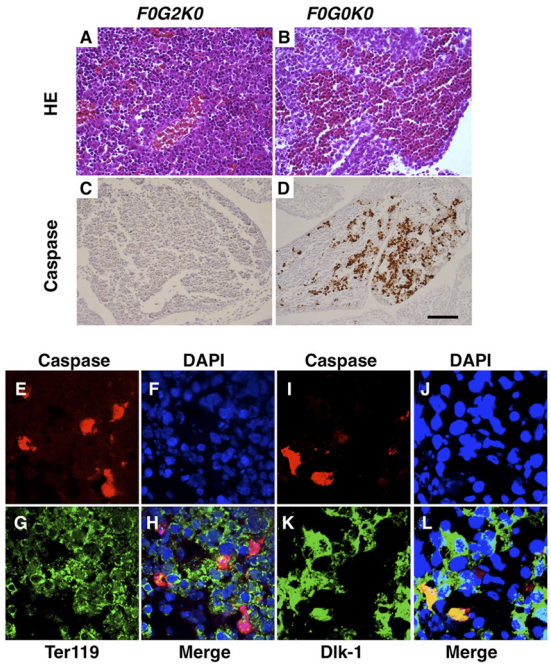

FIG 4 Apoptosis observed in fetal livers of *F0G0K0* embryos at E13.5. (A and B) Hematoxylin-eosin (HE) staining of fetal livers of *F0G2K0* and *F0G0K0* embryos. (C and D) Immunohistochemistry for cleaved caspase-3 in fetal livers of *F0G2K0* and *F0G0K0* embryos. Scale bar corresponds to 50 μm. (E to L) Double-immunofluorescence staining of cleaved caspase-3 (red) and Ter119 (green) or Dlk-1 (green). Nuclear DAPI (4′,6-diamidino-2-phenylindole) staining is shown in blue. The merge images of E to G and I to K are shown in panels H and L, respectively.

TABLE 3 Microarray analysis of ARE-dependent gene expression in *F0G2K0* and *F0G0K0* embryos

Gene product	Description	Fold increase in expression in embryos[a]: E10.5 *F0G0K0*/E10.5 *F0G2K0*	E10.5 *F0G0K0*/E9.5 *F0G2K0*
Gclc	Glutamylcysteine ligase, catalytic subunit	1.2	1.0
Gclm	Glutamylcysteine ligase, modulator subunit	1.6	1.9
Gpx1	Glutathione peroxidase 1	−1.1	1.0
Gpx2	Glutathione peroxidase 2	−1.2	1.0
Gsta3	Glutathione *S*-transferase A3	−1.5	−1.5
Gsta4	Glutathione *S*-transferase A4	1.0	1.0
Gstm1	Glutathione *S*-transferase M1	1.1	1.1
Gstm2	Glutathione *S*-transferase M2	−1.1	1.0
Gstm3	Glutathione *S*-transferase M3	−1.1	−1.1
Gstm4	Glutathione *S*-transferase M4	1.1	1.1
Gstm5	Glutathione *S*-transferase M5	1.0	1.0
Gstp1	Glutathione *S*-transferase P1	1.1	1.0
Hmox1	Heme oxygenase 1	2.6	2.7
Nqo1	NAD(P)H:quinone oxidoreductase	−1.8	−1.1
Prdx1	Peroxiredoxin 1	1.1	−1.1
Txnrd1	Thioredoxin reductase 1	1.0	−1.1

[a] Each value indicates the fold increase in the gene expression of *F0G0K0* embryos at E10.5 relative to that of *F0G2K0* embryos at E10.5 or *F0G0K0* embryos at E10.5 relative to that of *F0G2K0* embryos at E9.5.

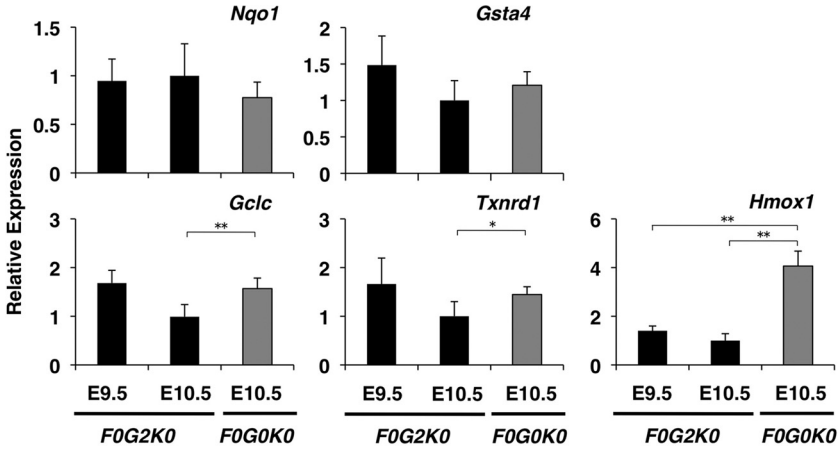

FIG 5 Expression profiles of antioxidant and xenobiotic-metabolizing enzyme genes in *F0G2K0* and *F0G0K0* embryos. Gene expression was examined by quantitative RT-PCR. The genotypes and gestational ages of the embryos are indicated. The expression level of each mRNA was normalized to the rRNA abundance. The average values of *F0G2K0* embryos at E10.5 are set to 1. Values are means ± standard deviations ($n = 3$ to 6). Student's *t* test was used to calculate statistical significance (*P*). *, $P < 0.05$; **, $P < 0.01$.

acting downstream of p45 NF-E2 (25), suggesting that small Maf deficiency affected p45 NF-E2-dependent gene expression (Fig. 6). However, these genes seem not to be the primary cause of the lethality, as *p45 NF-E2* knockout mice survive until birth (35). There are other genes that may cause the phenotype, such as genes involved in cell adhesion and receptor/channel and signal transduction (Fig. 6). Genes increased in *F0G0K0* embryos may also be associated with the phenotypes (see Fig. S1 in the supplemental material), since the Bach1-small Maf heterodimer is known to act as a repressor (36).

Rescue of small *Maf* triple-knockout mice by transgenic expression of *MafG*. It is important to note that the *F0G0K0* embryos examined in this study were derived from single lines of embryonic stem (ES) cells that were deficient for *MafF*, *MafG*, or *MafK*. Hence, there exists a formal possibility that unrelated mutations generated during the course of creating the mutants might affect the phenotype observed in *F0G0K0* embryos. To address this formal possibility and test the contention that the abnormalities observed in *F0G0K0* embryos are truly caused by the loss of small Mafs, we attempted a transgenic complementation rescue analysis. To this end, we decided to rescue the *F0G0K0* mice by transgenic expression of the MafG protein, since a single wild-type allele of *MafG* appeared to be sufficient to avoid embryonic lethality and to sustain fertility of adult mice.

To utilize the *MafG* gene regulatory region for transgene construct, we isolated genomic sequences including the *MafG* gene and examined their activity using a *LacZ* reporter gene to generate transgenic mice. We generated and examined transgenic mouse lines expressing the β-galactosidase reporter under the regulation of *MafG* (Fig. 7A). We found that a genomic segment covering 6.8 kbp upstream and 4.8 kbp downstream of *MafG* gene nicely recapitulated the endogenous *MafG* gene expression pattern, which could be assessed by the examination of the *LacZ* gene knock-in into the *MafG* locus (33). Accordingly, we designated this genomic region as *MafG* regulatory domain (*MGRD*). *MGRD*-mediated β-galactosidase expression was observed broadly in the transgenic embryos, and this was similar to the pattern observed in *LacZ*/*MafG* knock-in mice (Fig. 7B).

Finally, we generated transgenic mice expressing an exogenous

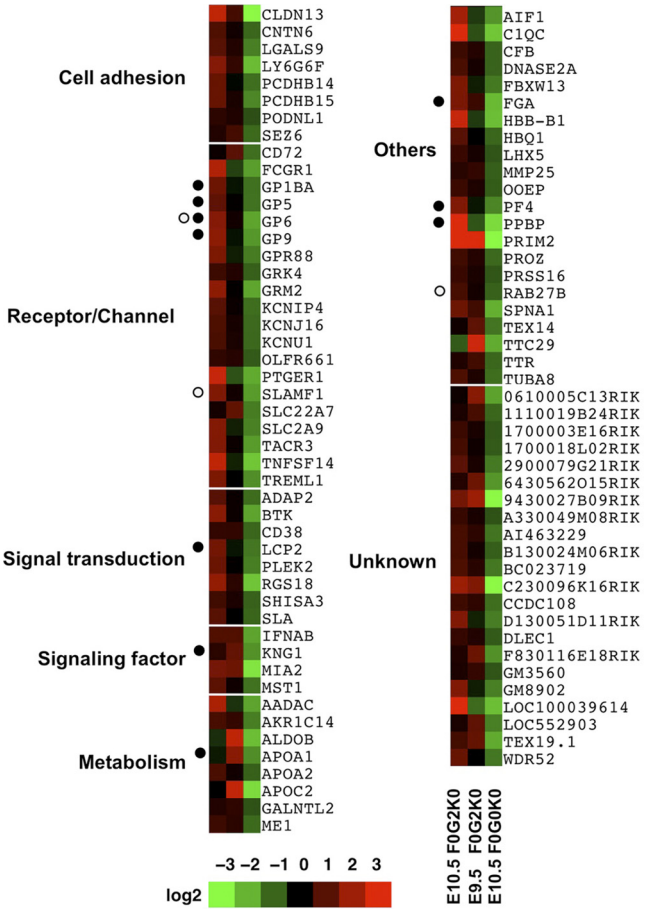

FIG 6 Heat map of relative expression levels of genes decreased in E10.5 *F0G0K0* embryos in comparison with both E10.5 and E9.5 *F0G2K0* embryos (over 2.0-fold). Heat map colors indicate normalized expression level ($\log_2$). Genes were categorized by function. Closed circles indicate genes involved in hemostasis according to pathway analysis. Open circles indicate genes that act downstream of p45 NF-E2 (25).

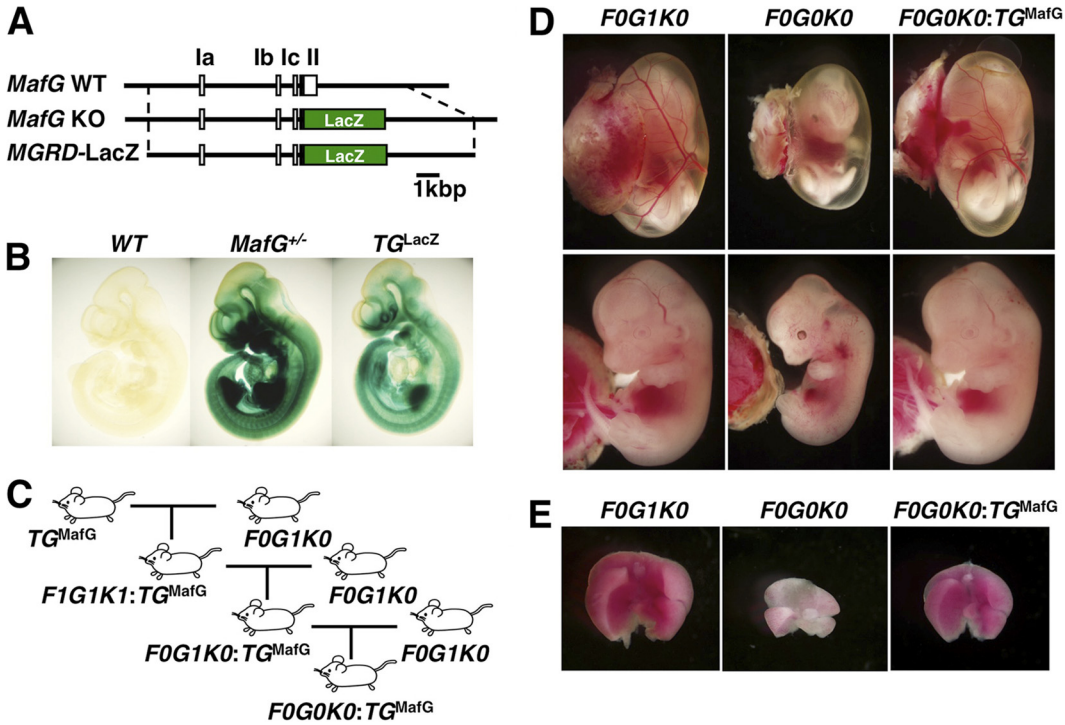

FIG 7 Small *Maf* triple-knockout mice were rescued by transgenic expression of *MafG*. (A) Schematic structure of the *MGRD-LacZ* transgene. The mouse *MafG* wild-type (WT) allele is depicted at the top. The *MafG* knockout (KO) allele is shown beneath the wild-type locus. (B) Whole-mount LacZ staining in WT, $MafG^{+/-}$, and TG^{LacZ} embryos at E10.5 (C) The mating strategy for producing *F0G0K0* mice rescued by TG^{MafG} mice (*F0G0K0*:TG^{MafG} mice). (D) Representative pictures of *F0G1K0*, *F0G0K0*, and *F0G0K0*:TG^{MafG} embryos at E13.5 with or without yolk sac. (E) Representative pictures of fetal livers for each genotype at E13.5.

MafG cDNA under the regulation of *MGRD* (TG^{MafG} mice) and bred the transgenic mice into an *F0G0K0* background. To this end, several crosses of TG^{MafG} mice with *F0G1K0* mice were conducted, as shown in Fig. 7C. At embryonic stages, rescued embryos (*F0G0K0*:TG^{MafG} mice) exhibited normal growth and liver development (Fig. 7D and E). No signs of anemia or growth retardation were observed. Moreover, rescued mice were born at the expected Mendelian frequency and showed no behavioral or hematological abnormalities (data not shown). Taken together, these results demonstrate that the E13.5 embryonic lethality and liver dysplasia observed in *F0G0K0* embryos are a result of the loss of function of all small Mafs in the mouse.

DISCUSSION

The CNC family of transcription factors activates unique sets of target genes through heterodimerizing with small Mafs. Contributions of the CNC proteins to the expression of ARE-dependent cytoprotective genes have been demonstrated by means of loss-of-function and/or gain-of-function analyses based on the mouse genetics (3, 11, 19, 25, 39). However, the contributions of small Mafs to those partnerships have not been addressed *in vivo*. Therefore, in this study we examined the phenotypes of *F0G0K0* mice and delineated how small Mafs participate in ARE-dependent cytoprotective gene regulation. While the small *Maf* triple-knockout (*F0G0K0*) mice appeared to develop normally up to E8.5, the embryos exhibited severe growth retardation, and all embryos died by E13.5. The *F0G0K0* embryos showed severe liver hypoplasia, and expression of ARE-dependent cytoprotective genes in the liver was profoundly reduced. Contrary to the observation in E13.5 livers, most ARE-dependent cytoprotective genes were normally expressed in E10.5 *F0G0K0* embryos. These results thus demonstrate that the small Mafs are essential for embryonic development after E9.5, especially for the development of the fetal liver.

This study provides the first evidence that the small Mafs are required and functional during mid-embryogenesis. Since none of the small *Maf* gene single- or double-knockout mice exhibit embryonic lethality (23, 28, 29, 33), it has been assumed that small Mafs are functionally redundant during embryonic stages. Even if individual small *Maf* genes are required to drive transcription of critical target genes during embryogenesis, the other small Mafs complement the loss of function of any individual small Maf protein. However, in adult animals, MafG is the only small Maf protein enabling mice to survive without severe abnormalities (Fig. 1) (23). Furthermore, we showed in this study that *F0G1K0* mice bearing only a single active *MafG* allele are fertile to maturity (Fig. 1). The reason for the differences in the activities of the small Mafs is currently unknown. One plausible explanation may be due to the differences in the expression profiles of small *Maf* genes, as the *MafG* gene shows the broadest expression profile among the small *Maf* genes (28). These observations thus support the notion that MafG is the most critical of the small Mafs in adult stages.

As summarized in Fig. 8, the phenotypes of the small *Maf* triple-knockout embryos are very similar to those observed in *Nrf1 Nrf2* double-knockout embryos that exhibit growth retardation and die between E9.5 and E13.5 (19). This observation supports the notion that Nrf1 and Nrf2 require small Mafs as obliga-

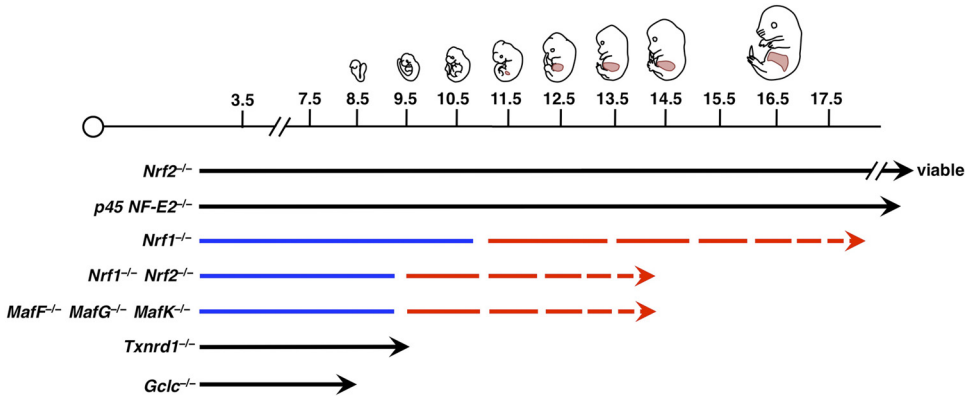

FIG 8 Comparison of phenotypes in gene-targeting mice showing embryonic lethality. Mouse embryonic stages are shown at the top with schematic images of embryos (not in the correct scale). Fetal livers are depicted as the pink areas. Horizontal arrows indicate the survival periods of each knockout mouse. The blue line represents the period without abnormality, and the red dotted line means the period with abnormality, such as growth retardation. *Nrf2* knockout mice are viable and fertile, although induction of cytoprotective genes is impaired in these mice (11). The majority of *p45 NF-E2* knockout mice die during perinatal period (35). *Nrf1* knockout mice display growth retardation after E10.5 and die by E18.5 (2). Both *F0G0K0* and *Nrf1 Nrf2* double-knockout mice display growth retardation after E9.5 and die by E13.5 (19). *Trxrd1* knockout mice die around E9.5 (12). *Gclc* knockout mice die around E8.5 (34).

tory partners. Additionally, fetal liver hypoplasia is a common phenotype observed in both small *Maf* triple-knockout and *Nrf1* knockout embryos (2), suggesting the importance of Nrf1-small Maf heterodimers in the maintenance of fetal liver homeostasis. Interestingly, other stress-inducible transcription factors, such as RelA, c-Jun, and MTF-1, have been reported to be indispensable for the growth or maintenance of fetal mouse livers (1, 6, 7). Since the fetal liver may be an organ that suffers from a variety of multiple stresses, development of the organ may rely on many different stress-inducible pathways, including the CNC-small Maf heterodimer.

It should be noted that there are significant discrepancies in the phenotypes of the small *Maf* triple-knockout embryos and *Nrf1 Nrf2* double-knockout embryos. Whereas apoptosis was broadly observed in the *Nrf1 Nrf2* double-knockout embryos (19), severe apoptosis was only significant in the livers of *F0G0K0* embryos. The phenotypes of *F0G0K0* embryos are also milder than those of *Nrf1 Nrf2* double-knockout embryos. This may be due to the impairment of Bach1 functions in the small *Maf* triple-knockout mice. Bach1-small Maf heterodimers are known to negatively regulate the expression of *Hmox1* (36), and heme oxygenase-1 is derepressed in the *F0G0K0* embryos (15). Since heme oxygenase-1 is known to act as an antioxidant enzyme (20), the activation of *Hmox1* gene expression might alleviate the phenotypes of *F0G0K0* embryos compared to the *Nrf1 Nrf2* double-knockout embryos.

To our surprise, ARE-dependent cytoprotective genes were expressed at a comparable level to wild-type embryos in the small *Maf* triple-knockout embryos at E10.5. These cytoprotective genes include several critical genes whose disruption results in far more severe defects in embryogenesis than those in the small *Maf* triple-knockout embryos. For example, as summarized in Fig. 8, *Gclc* gene mutant mice die by E8.5 with defects in gastrulation (34), and *Txnrd1* knockout mice die by E10.5 with growth retardation and reduced proliferation (12). Thus, expression of these cytoprotective genes is indispensable for early embryogenesis, but this study clearly demonstrates that the expression of these genes is maintained in a small Maf-independent manner in E10.5 embryos. There may be two possibilities to explain this phenomenon. One is that Nrf1 and Nrf2 can induce the expression of ARE-dependent cytoprotective genes without forming heterodimers with small Mafs at this stage. The other is that in early to midgestation embryos, cytoprotective genes are not under the regulation of CNC-small Maf heterodimers, implying the presence of a multilayered regulatory system that controls the cytoprotective genes. However, it is important to note that severe growth retardation is already evident in E10.5 small *Maf* triple-knockout embryos. This observation suggests that by this stage the small Mafs contribute to the regulation of critical genes that are distinct from typical ARE-dependent cytoprotective genes. Understanding the function of small Mafs during this period of embryogenesis would be an important future objective.

In conclusion, we have demonstrated that early embryogenesis proceeds without the contribution of small Mafs, but small Mafs are essential for embryogenesis beyond E9.5, especially in fetal liver development. The livers of small *Maf* triple-knockout embryos show severe apoptosis, which resembles the livers of *Nrf1 Nrf2* double-knockout embryos. This study thus revealed that the small *Maf* triple-knockout embryos phenocopy the *Nrf1 Nrf2* double-knockout embryos, and these results further support the contention that CNC proteins require small Mafs as obligatory partner molecules.

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