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Gene-Environment Interactions Target Mitogen-activated Protein 3 Kinase 1 (MAP3K1) Signaling in Eyelid Morphogenesis*

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Maureen Mongan[‡], Qinghang Meng[‡], Jingjing Wang[‡], Winston W.-Y. Kao[§], Alvaro Puga[‡], and Ying Xia[‡§1]

From the Departments of [‡]Environmental Health and [§]Ophthalmology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0056

Background: Adverse health effects may result from the synergy between environmental exposures and genetic makeup.
Results: The interaction between dioxin exposure *in utero* and specific genetic lesions disrupts embryonic eyelid closure.
Conclusion: Genetic and environmental factors synergize to inhibit developmental signaling pathways.
Significance: Understanding the mechanisms of gene-environment interaction is crucial to identify the etiology of congenital diseases and to develop preventive strategies.

Gene-environment interactions determine the biological outcomes through mechanisms that are poorly understood. Mouse embryonic eyelid closure is a well defined model to study the genetic control of developmental programs. Using this model, we investigated how exposure to dioxin-like environmental pollutants modifies the genetic risk of developmental abnormalities. Our studies reveal that mitogen-activated protein 3 kinase 1 (MAP3K1) signaling is a focal point of gene-environment crosstalk. Dioxin exposure, acting through the aryl hydrocarbon receptor (AHR), blocked eyelid closure in genetic mutants in which MAP3K1 signaling was attenuated but did not disturb this developmental program in either wild type or mutant mice with attenuated epidermal growth factor receptor or WNT signaling. Exposure also markedly inhibited c-Jun phosphorylation in *Map3k1*$^{+/-}$ embryonic eyelid epithelium, suggesting that dioxin-induced AHR pathways can synergize with gene mutations to inhibit MAP3K1 signaling. Our studies uncover a novel mechanism through which the dioxin-AHR axis interacts with the MAP3K1 signaling pathways during fetal development and provide strong empirical evidence that specific gene alterations can increase the risk of developmental abnormalities driven by environmental pollutant exposure.

The genetic code is the blueprint of organogenesis. Gene mutations, sequence variations, and structural alterations of the chromatin are the common causes of birth defects. Most defects, however, do not have a clear-cut inheritance pattern but have complex etiologies involving environmental influences and gene-environment interactions. As such, although the gene alterations establish a vulnerable biological state, the diseases occur only when unfavorable environmental conditions are also present (1). The interplay between genes and the environment is essentially responsible for individual diversity, phenotype variability, and etiologic heterogeneity of a myriad of disorders during development. To date, methods to capture the non-additive effects of gene-environment interactions are still lacking. This has limited our ability to identify the causative agents for many severe, costly, and often deadly congenital diseases.

Embryonic eyelid closure is a major morphogenetic event of mammalian development and is regulated by hundreds of genes (2). In mice, the eyelid closes between gestation day (GD)[2] 15.5 and GD16.5 as the result of elongation, spreading, forward movement, and ultimately fusion of the epithelium at the eyelid leading edge (3, 4). Failure of lid closure leads to a remarkable "eye open at birth" phenotype that is associated with ocular abnormalities that resemble blepharoptosis, strabismus, and congenital corneal diseases (5). More than 145 genetic mutant strains display an eye open at birth phenotype. Studies of the mutants have identified key signaling pathways, including MAP3K1, EGFR, WNT, bone morphogenetic proteins/activin B, Sonic hedgehog, and NOTCH, in the regulation of coordinated cell movement and epithelium morphogenesis (2, 6).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an organochlorinated pollutant and the prototype of hundreds of ubiquitous environmental compounds known collectively as dioxin-like chemicals (DLCs). DLCs are highly toxic and are released into the environment as by-products of incomplete combustion of fossil fuel and wood and incineration of municipal and industrial wastes. These chemicals are persistent in the environment and accumulate in soil, food, water, and wildlife, and the general population is exposed through ingestion of contaminated food and water. Because pregnant women transfer a fraction of their body burden to fetuses, the developmental toxicity of dioxin has been a serious concern (7). Notwithstanding the fact that the

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[1] To whom correspondence should be addressed: Dept. of Environmental Health, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0056. Tel.: 513-558-0371; E-mail: ying.xia@uc.edu.

[2] The abbreviations used are: GD, gestation day; MAP3K1, mitogen-activated protein 3 kinase 1; EGFR, epidermal growth factor receptor; GAB, Grb2-associated binder; DKK, Dickkopf; AHR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCB, polychlorinated dibenzofuran; DLC, dioxin-like chemical; CYP1A, cytochrome P450, family 1, member A; K12, keratin 12.

TABLE 1
List of chemical mixture used in this study

Chemicals	Planarity	IUPAC no.	Dose/kg	Toxic equivalency factor[a]
			mg	
3,3′,4,4′-Tetrachlorobiphenyl	Coplanar	77	50	0.0005
3,3′,4,4′,5-Pentachlorobiphenyl	Coplanar	126	0.25	0.1
3,3′,4,4′,5,5′-Hexachlorobiphenyl	Coplanar	169	2.5	0.03

[a] Listed in Van den Berg *et al.* (59).

epidemiological causative association between dioxin exposure and birth defects is yet to be established, studies in model systems have shown that TCDD is a potent teratogen (8, 9). In mice, TCDD exposure *in utero* causes developmental abnormalities, including but not limited to hydronephrosis, cleft palates, and vaginal thread formation (7). Some of the defects, such as cleft palates, occur at high incidence in children born in areas with high DLCs due to industrial, natural, or accidental release (10, 11).

In this work, we explored the genetic influences of dioxin exposure in the embryonic eyelid closure model and identified a novel gene-environment interaction mechanism in TCDD teratogenicity. We show that although TCDD exposure *in utero* did not affect eyelid development in wild type mice it blocked eyelid closure when MAP3K1 signaling was attenuated by gene mutation. Our data provide empirical evidence that specific pre-existing genetic conditions can increase the risk of adverse pregnancy outcomes of exposure to environmental agents.

Experimental Procedures

Chemicals, Reagents, and Antibodies—TCDD was purchased from Accustandard (New Haven, CT) and polychlorinated biphenyl (PCB) congeners 77, 126, and 169 (see Table 1) were purchased from ULTRA Scientific (North Kingstown, RI) and dissolved in corn oil. The antibodies for cytochrome P450, family 1, member 1A1 (CYP1A1) were from Alpha Diagnostic International (San Antonio, TX). Anti-α-smooth muscle actin was from Abcam (Cambridge, MA), anti-β-actin was from Sigma, anti-c-Jun and anti-phospho-c-Jun were from Cell Signaling Technology (Beverly, MA), and anti-keratin 12 (K12) was described before (12). X-gal, Harris hematoxylin solution, alcoholic eosin Y solution, and colchicine were from Sigma, and the Alexa Fluor-conjugated secondary antibodies were from Invitrogen.

Mouse Colonies and Dosing—The *Map3k1*$^{+/-}$ mice were as described (13) and were backcrossed onto the C57BL6/J background for 10 generations, resulting in >99.9% C57BL6/J genomes in the knock-out line. The genetic mutant strains for *Dkk2*, *Egfr*^{F}, and *Gab1*^{F} were described before (14–16). The *Egfr*^{F} and *Gab1*^{F} were crossed with *Le-cre* mice to delete genes specifically in the ocular surface ectoderm on GD9.5 (5, 17). Mouse mating, handling, and genotyping used standard protocols.

Pregnant dams were treated on various GDs by oral gavage with either corn oil (vehicle) or chemicals dissolved in corn oil. Mice were housed in a vivarium accredited by the Association for Assessment and Accreditation of Laboratory Animal Care; the animals were treated humanely and with regard for alleviation of suffering. All experiments involving mice were conducted in accordance with the National Institutes of Health standards for the care and use of experimental animals and were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Biohazard Precaution—TCDD and many dioxin-like chemicals are toxic compounds and probable human carcinogens; all personnel were therefore instructed in safe handling procedures. Lab coats, gloves, and masks were worn at all times, and contaminated materials were collected separately for disposal by the Hazardous Waste Unit or by independent contractors. The carcasses of chemical-pretreated mice were treated as contaminated biological materials.

Histology, X-gal Staining, Immunohistochemistry, and Western Blotting Analyses—For histology and immunohistochemistry, the embryonic/fetal heads were fixed in 4% paraformaldehyde at 4 °C overnight. The tissues were embedded in Optimal Cutting Temperature compound and frozen or in paraffin. The entire eye was processed for sagittal sections at 5–8 μm. For complete histological evaluation, H&E staining was performed on three consecutive sections at every 15 sections throughout the eye, and images were captured using a Zeiss Axioplan 2 microscope. Immunohistochemistry was performed as described before using specified antibodies (18). Whole mount X-gal staining and immunostaining were performed as described previously (13). The β-gal activities were determined by β-Glo assay following the manufacturer's protocol (Promega, Madison, WI).

Fetal liver and skin were homogenized in lysis buffer as described before (19). The lysates were applied to sodium dodecyl sulfate (0.1%)-polyacrylamide (10%) minigels and transferred to nitrocellulose. Western blotting analysis was performed using the antibodies indicated.

Statistical Analyses—Statistical comparisons were performed with Student's two-tailed paired t test. Values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant.

Results

TCDD Exposure Synergies with Mutants of Map3k1, but Not Wnt or Egfr, in Perturbing Embryonic Eyelid Closure—The eyelid starts to close between GD15.5 and GD16.5. Following a morphogenetic process involving epithelial cell elongation, intercalation, and migration, the upper and lower eyelids fuse to cover the ocular surface (20) (Fig. 1*A*). Eyelid closure depends on the MAP3K1, WNT, and EGFR signaling pathways, and pathway inactivation through homozygous mutation of *Map3k1* and *Dkk2* in the whole body and *Egfr* and *Gab1* in ocular surface epithelium leads to the open eye defects (13, 16, 21–25). The heterozygous mutants, however, have normal eyelid closure, underscoring the recessive nature of the mutant alleles.

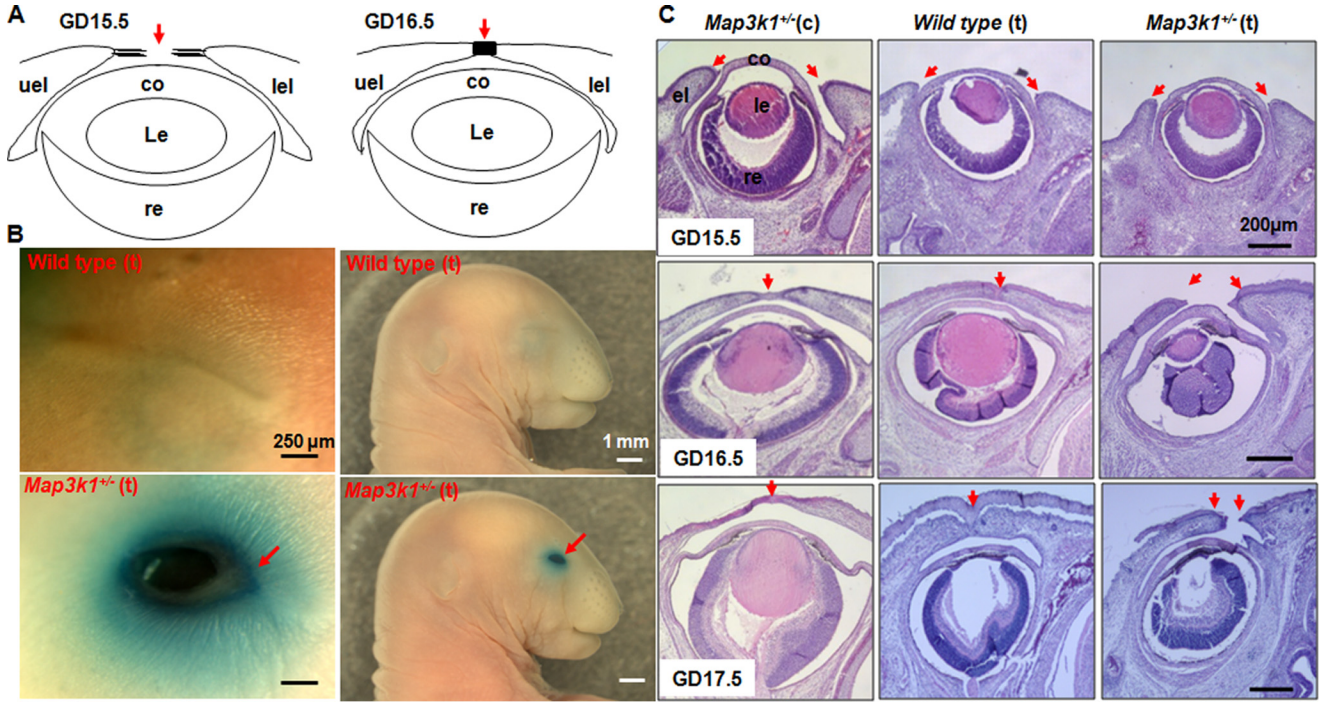

FIGURE 1. ***In utero* TCDD exposure induces the eye open phenotype in *Map3k1*$^{+/-}$ fetuses.** *A*, a sagittal view illustrating the developing eye prior to (GD15.5) and after (GD16.5) eyelid closure. *le*, lens; *uel*, upper eyelid; *lel*, lower eyelid; *co*, cornea; *re*, retina. *B*, the GD17.5 fetuses were collected from TCDD-exposed (*t*) dams and subjected to X-gal staining. Photographs were taken at high (*left panels*) and low (*right panels*) magnifications. The eyelid opening margin was defined by a pronounced X-gal staining due to the expression of MAP3K1-β-gal fusion protein. *C*, the GD15.5–17.5 fetuses collected from unexposed (*c*) or TCDD-exposed (*t*) dams were subjected to H&E staining and histological analyses. *Arrows* point at the eyelid leading edge and fusion junction. TCDD was applied as a single dose (50 μg/kg) by gavage on GD12.5 of pregnancy.

As suspected in most complex diseases, the mutant alleles may not produce a phenotype by themselves, but they increase the susceptibility to injury by environmental stressors. To test this hypothesis, we treated the pregnant dams carrying wild type and heterozygous embryos with TCDD at 12.5 days post-coitus and examined the eyelids in GD17.5–18.5 fetuses. The exposed wild type, *Dkk2*$^{+/-}$, *Egfr*$^{+/\Delta OSE}$, and *Gab1*$^{+/\Delta OSE}$ fetuses had fully closed eyelids indistinguishable from the unexposed fetuses (Table 2 and data not shown). In striking contrast, most exposed *Map3k1*$^{+/-}$ fetuses displayed an open eye phenotype (Fig. 1*B*).

To determine whether the open eye was due to failure of eyelid closure or to premature eyelid opening, we performed a histological examination of fetuses at different developmental stages. Regardless of genotype and exposure to TCDD, all GD15.5 fetuses had widely opened eyelids that were morphologically identical (Fig. 1*C*). The GD16.5 fetuses had the upper and lower eyelids fused in unexposed *Map3k1*$^{+/-}$ and exposed wild type but had the eyelid partially open in TCDD-treated *Map3k1*$^{+/-}$ fetuses. Thus, the open eye phenotype in the TCDD-exposed *Map3k1* hemizygotes was the result of defective eyelid closure.

The Dose and Developmental Window of TCDD Toxicity—Exposure to TCDD on different GDs could produce a varying magnitude of responses (26). To explore the time window of eyelid development vulnerability to exposure, we treated the pregnant dams with a single dose of 50 μg/kg TCDD on GD12.5, GD13.5, or GD14.5 (Fig. 2*A*). Although all *Map3k1*$^{+/-}$ GD17.5 fetuses displayed the open eye phenotype when TCDD was administered on GD12.5, 30% of the fetuses had the phenotype when TCDD was administered on GD13.5, and none of the *Map3k1*$^{+/-}$ fetuses had detectable eyelid defects when TCDD was administered on GD14.5 (Fig. 2*A*).

TABLE 2
Eyelid development in genetic mutant mice

Genotype	Eyelid status (no. fetuses)	
	Open	Closed
Egfr$^{+/\Delta OSE}$	0	10
Egfr$^{\Delta OSE/\Delta OSE}$	6	0
Gab1$^{+/\Delta OSE}$	0	10
Gab1$^{\Delta OSE/\Delta OSE}$	6	0
Dkk2$^{+/-}$, TCDD	0	9
Egfr$^{+/\Delta OSE}$, TCDD	0	1
Gab1$^{+/\Delta OSE}$, TCDD	0	2

To evaluate the effective dose, we gavaged the pregnant dams with different TCDD doses and examined the fetuses on GD17.5. When administered on GD12.5, 50 μg/kg TCDD induced open eyes in all *Map3k1*$^{+/-}$ fetuses, whereas 25 μg/kg TCDD caused the phenotype in 40% of fetuses, and 5 μg/kg TCDD did not have an effect (Fig. 2*B*). When administered on GD13.5, 75, 65, and 50 μg/kg TCDD led to the open eye phenotype in 70, 50, and 10% of the *Map3k1*$^{+/-}$ fetuses, respectively (Fig. 2*C*).

Two or more dams under each treatment condition were examined, and the open eye phenotype was not correlated with a specific litter or litter size. It is important to note that none of the wild type fetuses ($n = 106$) exhibited defective eyelid development, and there was no overt maternal toxicity under any of the treatment conditions, consistent with previous reports (27). In addition, wild type and *Map3k1*$^{+/-}$ fetuses exposed to high doses of TCDD (25 or 50 μg/kg) exhibited cleft palates and

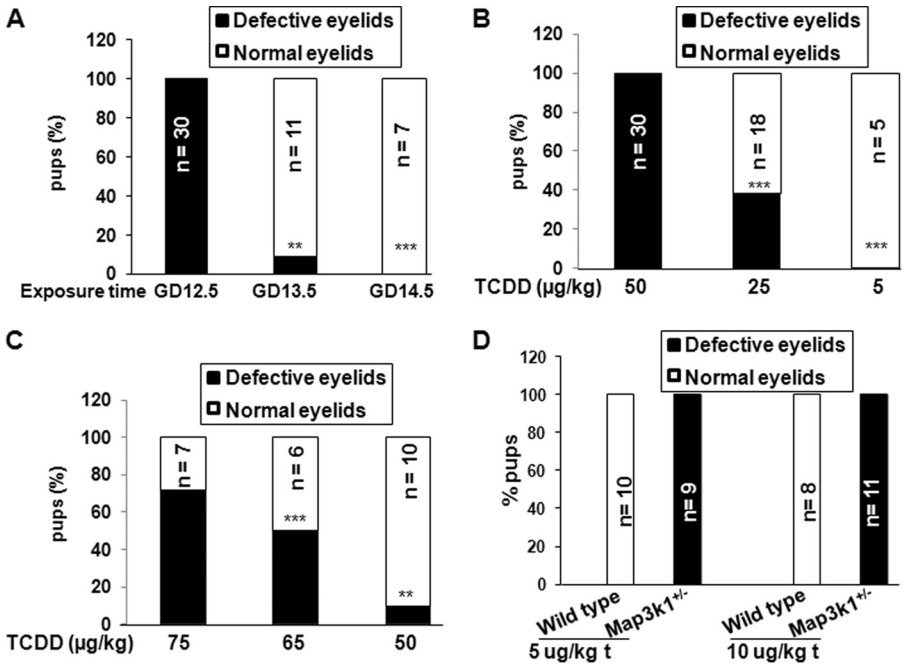

FIGURE 2. **Time and dose of TCDD exposure and eyelid development.** The dams were treated by gavage with a single dose of 50 μg/kg TCDD on various gestation days as indicated (*A*), and various doses of TCDD were administered at 12.5 (*B*) or 13.5 days (*C*) postcoitus. The penetrance of the eye open phenotype in *Map3k1*$^{+/-}$ fetuses was evaluated on GD17.5. *D*, the dams were gavaged with 5 or 10 μg/kg TCDD at 10.5, 11.5, and 12.5 days postcoitus. The eye phenotype was examined in wild type and *Map3k1*$^{+/-}$ GD17.5 fetuses. *n*, number of fetuses of a given genotype examined in two or more dams. The variability of TCDD responses was analyzed by comparing the percentage of affected pups/dam with those caused by 50 μg/kg TCDD at GD12.5. **, $p < 0.01$ and ***, $p < 0.001$ were considered significant.

hypoplastic Harderian glands (data not shown) comparable with the dosing conditions that induce the cleft palate phenotype established by others (28, 29).

Based on previous determinations using isotopically labeled TCDD, the above doses given to the pregnant dams are estimated to correspond to 1.7–25 ng per embryo (27) and are within the range of the reported mean human background body burdens for dioxin and dioxin-like compounds of ~9–13 ng of toxicity equivalent/kg (30). In contrast, real life environmental exposures are persistent, continuous, and long term. To mimic environmental exposure, we treated pregnant dams repeatedly on GD10.5, GD11.5, and GD12.5 with lower TCDD doses ranging from 2.5 to 10 μg/kg. The 2.5 μg/kg dose did not affect eyelid closure in the fetuses regardless of genotype, but the 5 and 10 μg/kg doses, although having no effect on eyelid closure in the wild type fetuses, caused the open eye phenotype in all of the *Map3k1*$^{+/-}$ fetuses (Fig. 2*D*).

Taken together, our data show that in addition to its toxicity in causing cleft palates, Harderian gland hypoplasia, and other developmental defects TCDD blocks embryonic eyelid closure when exposure occurs on or before GD12.5. The toxicity manifests itself only in the *Map3k1* hemizygotes, indicating that genetic susceptibility is a pre-requisite for TCDD toxicity in eyelid development.

Dioxin Toxicity Is Mediated by the Aryl Hydrocarbon Receptor (AHR)—Most biological effects of dioxin are mediated by the AHR, a ligand-activated basic helix-loop-helix-Per-Arnt-Sim transcription factor. To evaluate whether AHR activation was involved in eyelid development, we examined the effects of a mixture of PCBs, including PCB77, PCB126, and PCB169 (Table 1), which like TCDD are AHR agonists. The composition of the mixture was based on the ratio of these compounds in foodstuff and through the work of others has been established and accepted for environmental health research (31). We treated the pregnant dams repeatedly with the PCB mixture at GD11.5 and GD12.5 and examined fetuses at GD17.5. Similar to TCDD, the PCBs caused open eye phenotype in *Map3k1* hemizygotes but not in wild type fetuses (Fig. 3*A*).

To test genetically the role of AHR in TCDD-induced eyelid defects, we used fetuses from the cross of *Map3k1*$^{+/+}$*Ahr*$^{+/-}$ with *Map3k1*$^{-/-}$*Ahr*$^{+/-}$ mice. After TCDD exposure at GD12.5, the majority of *Map3k1*$^{+/-}$ GD17.5 fetuses had open eyes when the *Ahr* genotype was *Ahr*$^{+/+}$ or *Ahr*$^{+/-}$, but all of them had eyelids fully closed when it was *Ahr*$^{-/-}$ (Fig. 3, *B* and *C*). Although most *Map3k1*$^{+/-}$*Ahr*$^{+/-}$ fetuses had their eyes closed, a few had eyes slightly open (Fig. 3*C*).

Ligand binding induces AHR translocation from the cytoplasm to the nucleus where AHR heterodimerizes with the AHR nuclear translocator or interacts with other transcription factors (32). The nuclear AHR complexes cause highly cell-specific transcriptome changes, leading to biological end points that include developmental toxicity (33). The well established AHR transcription target is *Cyp1a1*, which codes for enzymes responsible for the metabolism and detoxification of exogenous chemicals and toxic responses (34). In the liver and skin of exposed fetuses, we detected a robust CYP1A1 induction in *Ahr*$^{+/+}$ and *Ahr*$^{+/-}$ but not *Ahr*$^{-/-}$ fetuses (Fig. 3*D*). Furthermore, the level of CYP1A1 was the same in wild type and *Map3k1* mutant mice, ruling out the possibility that MAP3K1 signaling is required for AHR activity in gene induction (Fig. 3*E*).

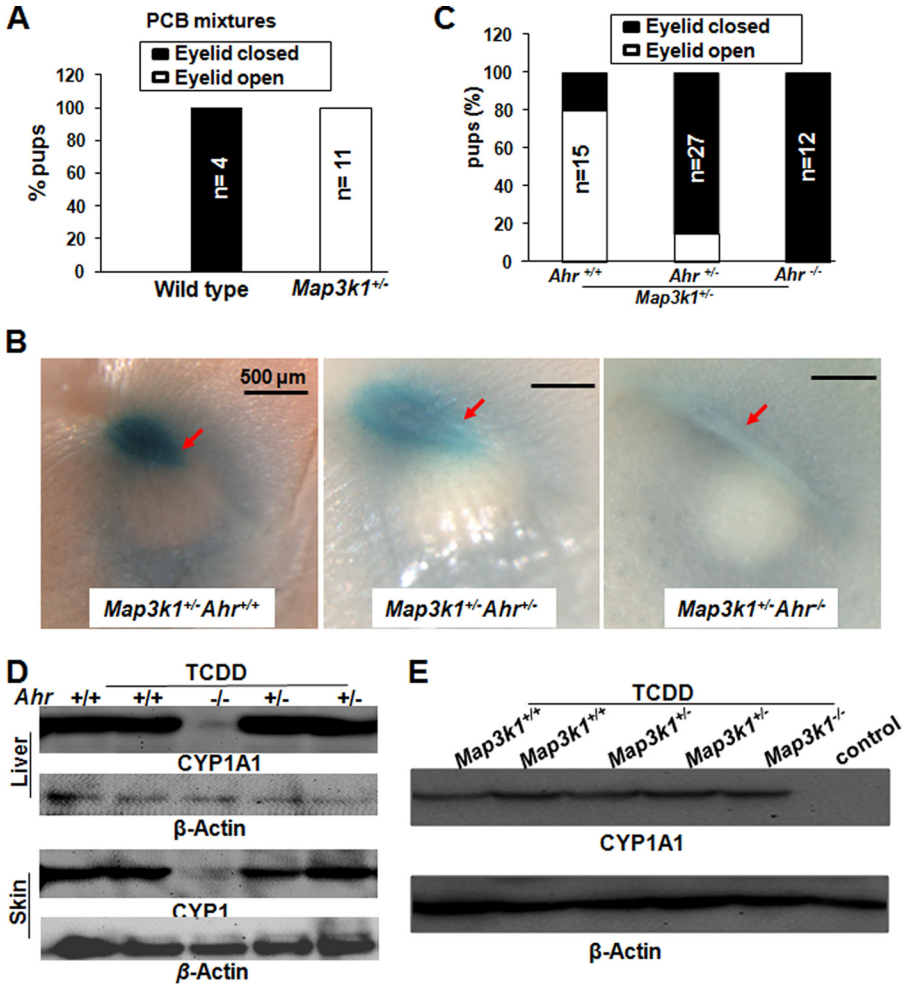

FIGURE 3. **AHR mediates TCDD effects on eyelid closure in *Map3k1*$^{+/-}$ fetuses.** *A*, dams carrying wild type and *Map3k1*$^{+/-}$ embryos were treated by gavage twice with the PCB mixtures on days 10.5 and 11.5 postcoitus. *B–E*, dams carrying *Map3k1* and *Ahr* mutant embryos were gavaged with 50 μg/kg TCDD on day 12.5 postcoitus. *A–C*, fetuses were collected at GD17.5, subjected to X-gal staining, and photographed, and the penetrance of the open eye phenotype was quantified. *Arrows* point at the eyelid margin. *n*, number of fetuses of the given genotype examined. The fetal liver and skin lysates (*D*) or liver lysates (*E*) were subjected to Western blotting for CYP1A1 and β-actin. Liver lysates from unexposed fetuses were used as a control in *E*.

TCDD Targets MAP3K1 Signaling—MAP3K1 is a member of the MAP3K superfamily responsible for activation of the MAP2K-MAPK cascades. In the developing eyelid, MAP3K1 is expressed abundantly in the epithelial cells and is required for activation of the Jun N-terminal kinases (JNKs), leading to phosphorylation of the transcription factor c-Jun (13). Previous genetic data have shown that polygenic lesions that act together to significantly inhibit MAP3K1 signaling can cause defective eyelid closure (13, 35, 36). For example, the *Jnk1*-null mutants have normal eyelid closure, but they display the eye open at birth phenotype in a *Map3k1*$^{+/-}$ genetic background in which MAP3K1 is reduced to half (35). Using the *Jnk1* mutant model, we tested whether TCDD exposure had an effect similar to that of *Map3k1* heterozygosity. Dams carrying *Jnk1* mutant E12.5 embryos were treated with TCDD, and the eyelids in E17.5 fetuses were examined. We found that although *Jnk1*$^{+/-}$ fetuses had relatively normal eyelid development all the *Jnk1*$^{-/-}$ fetuses displayed the "open eye" defects as expected (Fig. 4*A*). These observations suggest that TCDD exposure mimicked single *Map3k1* allele loss in causing eyelid defects in the *Jnk1*$^{-/-}$ fetuses (35).

To evaluate whether TCDD attenuated MAP3K1 expression, we performed whole mount X-gal staining of the *Map3k1*$^{+/-}$ fetuses and measured the expression of the endogenous MAP3K1-β-gal fusion protein (13). We detected abundant expression of β-gal in the eyelid leading edge with the intensity and pattern unaffected by TCDD exposure (Fig. 4*B*). Furthermore, in cultured *Map3k1*$^{+/-}$ cells, induction of MAP3K1-β-gal expression by colchicine-mediated microtubule disruption was unaffected by pretreating the cells with TCDD for 1–3 days, leading to the conclusion that TCDD does not affect MAP3K1 expression (Fig. 4*C*).

Alternatively, TCDD could attenuate MAP3K1 activity in the activation of the JNK-c-Jun cascades (35). To evaluate this possibility, we examined the expression and phosphorylation of c-Jun in the GD15.5 eyelids. Compared with untreated fetuses, the TCDD-treated fetuses had significantly reduced c-Jun phosphorylation in the eyelid tip epithelium, whereas c-Jun expression was unaffected (Fig. 4, *D* and *E*). Approximately 50 and 25% of c-Jun was phosphorylated in wild type fetuses of unexposed or TCDD-exposed dams, respectively. However, only 10% phospho-c-Jun was detected in *Map3k1*$^{+/-}$ fetuses of TCDD-

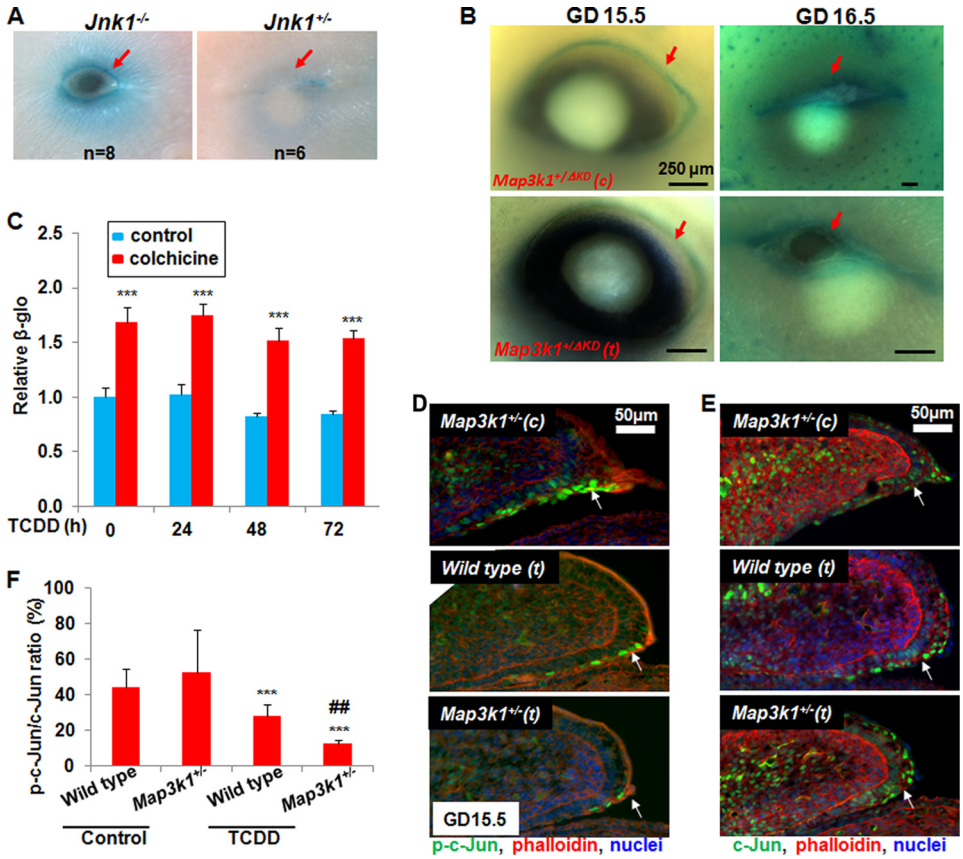

FIGURE 4. **TCDD targets the MAP3K1 pathways.** *A*, dams carrying *Jnk1* mutant embryos were treated with TCDD (50 μg/kg) at GD12.5, fetuses were collected at GD17.5, and eyelids were stained and photographed. *n*, number of eyes examined for the given genotype. *B*, GD15.5 and GD16.5 *Map3k1*$^{+/-}$ fetuses with or without 50 μg/kg TCDD exposure on GD12.5 were subjected to whole mount X-gal staining, a method detecting the MAP3K1-GAL fusion protein. *Red arrows* point at the eyelid margin. *C*, *Map3k1*$^{+/-}$ mouse embryonic fibroblasts were treated with TCDD for various lengths of time. Colchicine-mediated induction of MAP3K1-β-gal fusion protein was determined by β-Glo assays. *D–F*, immunohistochemistry staining of the GD15.5 embryonic eyelids showed abundant c-Jun phosphorylation (*D*) and expression (*E*) in epithelial cells at the eyelid leading edge. *F*, quantification of immunostaining-positive signals in *D* and *E*. Exposure to TCDD did not alter c-Jun expression but inhibited c-Jun phosphorylation in wild type and more so in *Map3k1*$^{+/-}$ embryos. Statistical analyses were performed by comparing the phospho (*p*)-Jun/Jun levels in control *versus* TCDD (***, $p < 0.001$) and in TCDD-treated wild type *versus* *Map3k1*$^{+/-}$ (##, $p < 0.01$). In unexposed (*c*) and TCDD-exposed (*t*) samples, *white arrows* point at the staining-positive cells. *Error bars* represent S.D.

exposed dams. Hence, TCDD acts synergistically with *Map3k1* allelic lesions to inhibit the signaling pathways that lead to c-Jun phosphorylation.

Ocular Abnormalities Associated with TCDD Exposure and Map3k1 Allelic Lesions—Eyelid closure in embryogenesis provides morphological support for the development of ocular adnexal structures (5). The prenatal mouse fetuses with defective eyelid closure display truncation of the eyelid tarsal muscles, which are responsible for eyelid elevation. The α-smooth muscle actin-positive tarsal muscles were indeed truncated in exposed *Map3k1*$^{+/-}$ fetuses, corresponding to defective eyelid closure, whereas the tarsal muscles extended continuously into the upper and lower eyelids in unexposed *Map3k1*$^{+/-}$ and exposed wild type fetuses (Fig. 5*A*).

Although eyelid closure resulting in the formation of the conjunctiva sac has been speculated to be required for protecting the cornea during development, our previous data show that it is dispensable for corneal epithelium differentiation in prenatal fetuses (5, 37, 38). Consistent with this notion, K12, the cornea-specific keratin, was expressed in the corneal epithelium of all unexposed fetuses regardless of their eyelid closure status (5) (Fig. 5*B*). Interestingly, although K12 expression was still abundant in wild type fetuses exposed to TCDD, it was markedly reduced in the TCDD-exposed *Map3k1*$^{+/-}$ fetuses. These observations suggest that closed eyelids, although dispensable for corneal development in naïve conditions, may actually be required for protecting the immature corneas from injuries under adverse maternal conditions and environmental insults, such as exposure to TCDD.

Discussion

Using genetic mouse models, we present a case where a birth defect arises from the interplay between a genetic lesion and exposure to an environmental toxicant. We show that *Map3k1* allele loss increased the risk of eyelid malformation in fetuses exposed to dioxin and DLCs and likewise that dioxin exposure increased the risk of eyelid malformation due to *Map3k1* allelic loss or mutation. Although neither chemical exposure nor *Map3k1* hemizygosity alone had an adverse effect, their combination produced an open eye phenotype by blocking eyelid closure during embryogenesis. Importantly, TCDD did not synergize with allelic lesions of *Dkk2*, *Egfr*, and *Gab1* whose products are also essential for eyelid closure. Our data provide compelling evidence that specific gene variations can modify the effect of TCDD exposure.

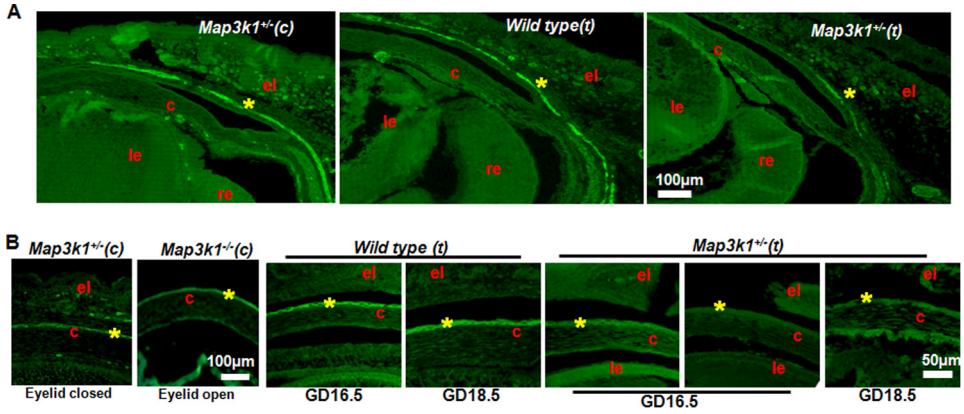

FIGURE 5. **Eye abnormalities resulting from the gene-environment interactions.** *A*, the eye sections of GD17.5 fetuses were subjected to immunohistochemistry staining using anti-α-smooth muscle actin antibodies (*green*). The eyelid tarsal muscles (*) were strong and continuous in untreated *Map3k1*$^{+/-}$ and treated wild type but were weak and truncated in treated *Map3k1*$^{+/-}$ mutants. *B*, eye sections were subjected to immunohistochemistry staining using anti-K12 (*green*). K12 expression was detected in corneal epithelium (*) of unexposed *Map3k1*$^{+/-}$ and *Map3k1*$^{-/-}$ and exposed wild type but was largely reduced in that of exposed *Map3k1*$^{+/-}$ fetuses, corresponding to defective eyelid closure. The unexposed (*c*) or TCDD-exposed (*t*) fetuses were as labeled. *le*, lens; *el*, eyelid; *c*, cornea; *re*, retina.

Like its teratogenicity in cleft palates and hydronephrosis, TCDD perturbs eyelid development through the dioxin receptor AHR. The open eye phenotype is less severe when the *Ahr* gene dosage is reduced by half and completely abolished when AHR is absent, suggesting an essential and dose-dependent role of AHR in TCDD toxicity. The AHR has been shown to be required for nervous system and eye development. The *Ahr* knock-out mice have acquired central nervous system deficits that lead to abnormal eye movements, and the knock-out retinas are more susceptible to age-related macular degeneration (39–41). In contrast, AHR itself is dispensable for eyelid development but is required for mediating the effects of TCDD. Specifically, TCDD acts through AHR to attenuate MAP3K1 signaling. The attenuation is small, but when aided by *Map3k1* heterozygosity, it leads to a significant reduction of c-Jun phosphorylation in eyelid epithelial cells. It appears that genetic and environmental insults can aggregate to reduce the MAP3K1 signal, and when this signal is reduced below a critical threshold, eyelid defects occur. Consistent with this idea, earlier genetic data have shown that polygenic lesions can also act additively to target the MAP3K1 network and cause the open eye phenotype (35, 36).

The mechanisms through which TCDD affects MAP3K1 signaling are not understood. One possibility is that genes regulated by the TCDD-AHR pathways can modulate the MAP3K1 signaling. In this context, AHR has been shown to target the VAV3-RHOA cascades through transcription-dependent and -independent mechanisms (42). RHOA can interact with MAP3K1 in actin stress fibers (43), and interestingly, genetic *RhoA* inactivation delays eyelid closure in the *Map3k1*$^{+/-}$ embryo/fetus (36). It is thus tempting to speculate that the TCDD-AHR axis targets the RHOA pathways, which in turn affect the MAP3K1-JNK-c-Jun cascades through transcription independent cross-talk of signaling pathways. Eyelid closure is likely one of the endogenous processes in which the TCDD-AHR pathways regulate epithelial cell migration and cytoskeleton reorganization. Hence, comparative analyses of TCDD-responsive genes in eyelid epithelial cells of wild type and *Map3k1*$^{+/-}$ fetuses may help to unveil the molecular and signaling mechanisms of TCDD in migration and morphogenesis.

DLCs are persistent pollutants found throughout the global environment, and all humans have background levels of exposure. Despite the utmost importance to public health, little is known about the genetic conditions relevant to the risk of exposure. Experiments in mice have identified genetic modifiers, including *Ahr*, *Cyp1a2*, *Egf*, *Raldh*, and *Rara*, for the developmental toxicity of dioxin (44–48). In the present report, we show that MAP3K1 signaling offers protection against TCDD toxicity. Mutation of genes along the pathway, *i.e. Map3k1* and *Jnk1*, render the eyelid developmental programs more susceptible to dioxin exposure. Along this line of research, the in-depth understanding of the molecular network through which MAP3K1 operates may lead to the identification of additional genetic risk factors and polygenic mechanisms underlying birth defects associated with dioxin exposure.

The fusion and reopening of upper and lower eyelids is a morphogenetic event conserved in mice and humans. Different from mice, eyelid closure and reopening in humans occur entirely *in utero* between 2 and 5 months of fetal life (3). Detection of human defects during this time is challenging, and as a consequence, the disease phenotypes associated with defective eyelid closure are largely unknown. Studies of genetic mutant mice with open eyelid phenotypes have provided an initial clue to the disease phenotypes as failure of lid closure in mice is linked to abnormalities of eyelid levator muscle and extraocular muscle (5). In addition to pathogenesis in the eyelid levator muscle, the exposed *Map3k1*$^{+/-}$ fetuses displayed abnormalities in corneal epithelium differentiation that were not observed in unexposed eye open at birth mutants. This is by far the first empirical evidence supporting Sevel's hypothesis suggesting that embryonic eyelid closure offers protection of the immature cornea from adverse maternal conditions and environmental insults (5, 38). Congenital anomalies of the cornea and eyelid tarsal muscles may have defective eyelid closure as the common underlying cause and *Map3k1* hemizygosity plus *in utero* dioxin exposure as a possible etiology.

In humans, recurrent missense mutations in the *MAP3K1* gene are found to be associated with cervical and breast cancers (49, 50). These mutants represent low penetrance susceptibility polymorphisms acting as modifier genes in patients who carry tumor suppressor mutation. In addition, germ line splice acceptor mutation of the *MAP3K1* allele has been associated with 46,XY gonadal dysgenesis (51, 52). The gain of function products of the mutant alleles lead to increased phosphorylation of p38 and ERK1/2 and binding with the cofactors RHOA and MAP3K4 (53). This results in shifting the balance of the sex-determining pathway. Interestingly, mice with the *Map3k1* gene inactivation have a normal appearance but display a minor testicular deficit in the developing gonad (54).

Despite its strong implications in eye development in mice, the *MAP3K1* mutation has not been linked to human eye diseases. It is worth noting, however, that large alterations of chromosomal regions in close proximity of the *MAP3K1* loci are found in sporadic cases of human congenital eye and cranial facial abnormalities (55, 56). Given that the genetic basis for most congenital eye diseases is still poorly understood, *MAP3K1* mutation may be one of the risk factors for unexplained congenital eye anomalies, an idea yet to be tested through extensive clinical genetic studies.

In light of the findings in mice, it is reasonable to speculate that diseases associated with human eyelid closure failure may have a multifactorial etiology that is inconsistent with simple Mendelian inheritance. For instance, among individuals with MAP3K1 allele lesions in the population, only those exposed prenatally to DLCs are likely to have congenital eye abnormalities. In this context, it is interesting to note that among people exposed to high doses of DLCs, such as the Yusho and Yu-Cheng PCB poison victims, aberrant eye development has been found in a few babies born to mothers exposed during pregnancy (57, 58). Whether the patients are individuals carrying recessive susceptible genetic lesions and thus have a low threshold for exposure-induced pathogenesis is a question to be addressed in the future.

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