Diphthamide modification on eukaryotic elongation factor 2 is needed to assure fidelity of mRNA translation and mouse development

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To study the role of the diphthamide modification on eukaryotic elongation factor 2 (eEF2), we generated an eEF2 Gly^{717}Arg mutant mouse, in which the first step of diphthamide biosynthesis is prevented. Interestingly, the Gly^{717}-to-Arg mutation partially compensates the eEF2 functional loss resulting from diphthamide deficiency, possibly because the added +1 charge compensates for the loss of the +1 charge on diphthamide. Therefore, in contrast to mouse embryonic fibroblasts (MEFs) from *OVCA1*$^{-/-}$ mice, *eEF2*$^{G717R/G717R}$ MEFs retain full activity in polypeptide elongation and have normal growth rates. Furthermore, *eEF2*$^{G717R/G717R}$ mice showed milder phenotypes than *OVCA1*$^{-/-}$ mice (which are 100% embryonic lethal) and a small fraction survived to adulthood without obvious abnormalities. Moreover, *eEF2*$^{G717R/G717R}$*/OVCA1*$^{-/-}$ double mutant mice displayed the milder phenotypes of the *eEF2*$^{G717R/G717R}$ mice, suggesting that the embryonic lethality of *OVCA1*$^{-/-}$ mice is due to diphthamide deficiency. We confirmed that the diphthamide modification is essential for eEF2 to prevent −1 frameshifting during translation and show that the Gly^{717}-to-Arg mutation cannot rescue this defect.

Eukaryotic elongation factor 2 (eEF2) is a member of the GTP-binding translation elongation factor family, and an essential factor for protein synthesis and cell survival. eEF2 drives the GTP-dependent translocation of the nascent polypeptide chain from the A site to the P site of the ribosome and advances mRNA by three bases during the elongation cycle of protein synthesis (1). eEF2 is highly homologous in all eukaryotes. In fact, eEF2 of humans, rats, mice, hamsters, and other mammals have exactly the same amino acid sequence. Intriguingly, all eukaryotic eEF2 proteins contain a unique posttranslationally modified histidine residue termed diphthamide (2, 3). Diphthamide modification occurs after eEF2 is translated and is irreversible, marking the completion of the biosynthesis of eEF2.

Although the physiological role of the diphthamide modification on eEF2 remains elusive, diphthamide is the well-known target for the adenosine diphosphate (ADP)-ribosylating toxins from bacterial pathogens, such as diphtheria toxin (DT) from *Corynebacterium diphtheriae*, *Pseudomonas* exotoxin A (ETA) from *Pseudomonas aeruginosa*, and the recently identified cholix toxin (CT) from *Vibrio cholerae* (4). As virulence factors, these ADP-ribosylating toxins catalyze transfer of the ADP ribose from nicotinamide adenine dinucleotide (NAD^+) to diphthamide on eEF2 (Fig. S1), thus inactivating eEF2, halting cellular protein synthesis, and causing cell death.

Because the diphthamide modification is required for the action of the ADP-ribosylating toxins, the complex diphthamide biosynthesis pathway is amenable to genetic analysis, and mutants defective in diphthamide biosynthesis have been isolated in both Chinese hamster ovary (CHO) cells and yeast (*Saccharomyces cerevisiae*) by selection for resistance to DT or an engineered ADP-ribosylating toxin – anthrax protective antigen (PA) + fusion protein 59 (FP59) (5–10). PA is the cellular binding moiety of anthrax toxin, which specifically binds to either capillary morphogenesis protein 2 (CMG2, major toxin receptor) or tumor endothelium marker 8 (TEM8, minor toxin receptor) on host cells (11–14) causing the endocytosis and translocation of anthrax lethal factor (LF), edema factor, or FP59 into the cytosol of host cells. FP59 is a fusion protein of LF amino acids 1–254 and the catalytic domain of *P. aeruginosa* exotoxin A (15) that kills cells by ADP ribosylation of eEF2 after delivery to cytosol by PA. Using the toxin-resistant mutant cells combined with a genetic complementation or gene-trapping approaches, many proteins required for diphthamide biosynthesis have been identified in eukaryotes from yeast to humans, including OVCA1 (ovarian cancer 1, identical to Dph1), Dph2, Dph3, Dph4, Dph5, and WDR85 (YBR246W in yeast) (16, 17). The biosynthesis of diphthamide represents one of the most complex posttranslational modifications, attesting to the importance of the diphthamide modification in eEF2 normal physiology (18). Surprisingly, none of the diphthamide-deficient mutants identified in either yeast or CHO cells exhibit strong phenotypes (5, 6, 8–10, 18, 19), demonstrating that the diphthamide modification is not essential for cell survival. However, recent studies of OVCA1 (Dph1), Dph3, and Dph4 knockout mice have shed light on the physiological role of diphthamide in multicellular organisms (20–22). Remarkably, OVCA1-, Dph3-, and Dph4-null mice die during embryonic development, suggesting an important role of diphthamide in embryonic development. Of these mutant mice, Dph3-null mice show a 2-d delay in development and die before embryonic day 12 (E12), whereas OVCA1- and Dph4-null mice phenocopy each other and show a 1-d delay in development, preaxial polydactyly, and die before or shortly after birth. It is not surprising that Dph3-null mice have more severe phenotypes because Dph3 is also involved in crucial tRNA anticodon modifications and is a functional component of the transcription Elongator complex (23, 24). Interestingly, OVCA1 is a previously identified tumor suppressor that plays an important role in regulation of cell proliferation and tumorigenesis (20).

Defects in diphthamide modification and resistance to the ADP-ribosylating toxins can also result from structural gene mutations in eEF2 (25–28). Among these mutations, the eEF2 Gly^{717}-to-Arg (G717R) mutation in CHO cells, resulting from a single base pair change from G to A at the first base of codon 717 in the eEF2 gene, is frequently obtained because the mutation has little effect on eEF2 activity in protein synthesis (25–27). Biochemical analyses demonstrated that the Gly^{717}-to-Arg

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mutation prevents the first step in diphthamide biosynthesis (26, 27), presumably by interfering with recognition and modification of the nearby His^{715} by the Dph enzymes.

To explore the roles of diphthamide in normal physiology and to avoid the possible issue of diphthamide biosynthesis proteins having other biological functions (as confirmed in the case of Dph3), we generated an eEF2 G717R mutant mouse, in which the mutated eEF2 lacks the diphthamide modification, even though the diphthamide biosynthesis machinery is intact. We found that the G717R mutation (having a gain of +1 charge) partially compensates the eEF2 functional loss resulting from diphthamide deficiency (which is accompanied by a loss of +1 charge). We show that $eEF2^{G717R/G717R}$ mouse embryonic fibroblasts (MEFs) retain full activity in polypeptide elongation and normal growth rates, and that a small fraction of the mice survive to adulthood without obvious abnormalities. Other characteristics of these mice are also described.

Results

Generation of $eEF2^{G717R}$ Mutant Mouse. Diphthamide deficiency can be induced by deletion of the genes involved in the diphthamide biosynthesis (20–22). However, these genes may have pleiotropic functions, as seen with Dph3 involvement in tRNA anticodon modifications (23). Therefore, to explore the physiological functions of diphthamide without interrupting other biological processes, we chose to introduce a point mutation by changing Gly^{717} to Arg ($eEF2^{G717R}$) in the mouse eEF2 locus. Both the Gly^{717} residue and the diphthamide^{715} are at the tip of eEF2 domain IV. In searching for CHO mutants that are resistant to the bacterial ADP-ribosylating toxins, we and others have found that the G717R mutation is the most commonly obtained mutation in the eEF2 structural gene, and one that has little effect on eEF2 activity (26–28). The G717R mutation blocks the first step of diphthamide modification on eEF2 (26, 27), making the cells resistant to the ADP-ribosylating toxins DT and PA + FP59. To generate the $eEF2^{G717R}$ mouse, we constructed a gene targeting vector containing the G717R mutation (Fig. 1 *A* and *B*) and used homologous recombination to generate mouse ES cells with one of the eEF2 alleles replaced by the mutated sequence (Fig. 1*B*). Two resulting ES cell clones with the desired mutation ($eEF2^{+/G717R}$) were obtained and used for injection into mouse blastocysts to generate chimeric mice. Germ line transmission of the eEF2 G717R mutation was achieved with chimeras from these clones, resulting in the heterozygous $eEF2^{+/G717R}$ mice (Fig. 1 *C* and *D*).

***eEF2*$^{G717R/G717R}$ Mice Are Delayed in Development.** To explore the in vivo roles of the diphthamide modification on eEF2, we set up $eEF2^{+/G717R}$ intercrosses to produce homozygous $eEF2^{G717R/G717R}$ mice. Genotyping of the offspring at the weaning age (3 wk old) revealed that only a small fraction were $eEF2^{G717R/G717R}$ mice (1.2% vs. the projected 25%, Table S1). Subsequently, staged embryos from the heterozygous intercrosses were analyzed. We found that the $eEF2^{G717R/G717R}$ mice were delayed in development after E14.5, displaying similar sizes as the WT and heterozygous littermates at E14.5, but significantly smaller sizes at E17.5 (Fig. 1 *E* and *F*). ADP-ribosylation analyses of the embryo tissue lysates were performed by detecting the incorporation of the biotin-labeled NAD^+ into eEF2 after 30-min incubation of the tissue lysates with DT. Whereas the eEF2 in WT tissue lysates was efficiently ADP ribosylated, ADP ribosylation in $eEF2^{G717R/G717R}$ tissues could not be detected (Fig. 1*G*). The eEF2 ADP-ribosylation level in $eEF2^{+/G717R}$ embryos was approximately half of that in $eEF2^{+/+}$ embryos, demonstrating that the WT and mutated alleles in the heterozygous embryos were equally expressed (Fig. 1*G*). These results are in line with previous findings that the G717R mutation in eEF2 results in deficiency of diphthamide modification on eEF2 (26–28). Histology analyses did not identify apparent abnormalities in most of the $eEF2^{G717R/G717R}$ embryos, although one case of s.c. hemorrhaging was observed. We found that most of the $eEF2^{G717R/G717R}$ mice died shortly after birth, likely due to premature birth from developmental delay. The heterozygous mice did not show any phenotypic abnormality. The results showing $eEF2^{G717R/G717R}$ mice are delayed in embryo development, together with the previously described embryonic lethality of OVCA1-, Dph3-, and Dph4-null mice, suggest that the diphthamide modification on eEF2 plays a crucial role in mouse development.

$OVCA1^{-/-}$ and $Dph4^{-/-}$ mice phenocopied each other with a delay in embryo development starting as early as E8.5, and having 100% prenatal lethality (20–22) (Table S2). Interestingly, the $eEF2^{G717R/G717R}$ mice displayed a milder phenotype, with a small fraction surviving to adulthood. Those $eEF2^{G717R/G717R}$ mice that survived birth subsequently appeared healthy and displayed a growth rate comparable to their WT and heterozygous littermates (see the parallel growth curves shown in Fig.

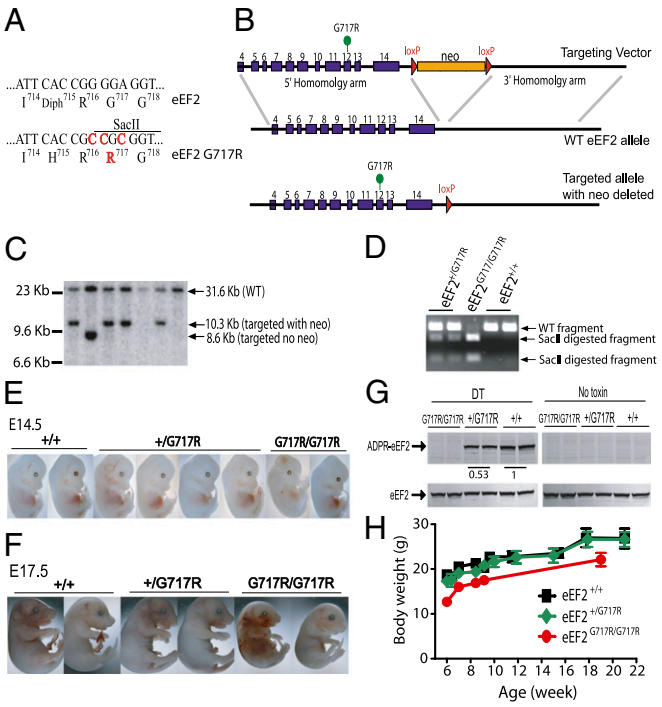

Fig. 1. The $eEF2^{G717R/G717R}$ mice are delayed in development. (*A*) The eEF2 G717R point mutation introduced into the mouse eEF2 locus. The mutation also generates a SacII site to facilitate genotyping as shown in *D*. Diphthamide is assembled on residue H^{715}. (*B*) Gene targeting of eEF2 by homologous recombination using the gene targeting vector containing the eEF2 G717R mutation. The Neo cassette flanked by two loxP sites was removed by breeding the resulting targeted mice with Cre general deleter mice. (*C*) Representative Southern blot analyses of offspring of eEF2-targeted mice. Genomic DNA digested with EcoRV was hybridized with a 3′ external probe. (*D*) Representative PCR genotyping analyses. A DNA fragment spanning the mutation site (with SacII site introduced, *A*) was amplified and digested by SacII, and separated on agarose gel. Note that the mutated fragment was digested into two smaller fragments, whereas the WT fragment remained intact. (*E* and *F*) Embryos at E14.5 (*E*) and E17.5 (*F*) with various genotypes. Note that $eEF2^{G717R/G717R}$ embryos at E14.5 are similar in size to WT and $eEF2^{+/G717R}$ embryos but significantly smaller at E17.5. (*G*) ADP-ribosylation analyses of $eEF2^{+/+}$, $eEF2^{+/G717R}$, and $eEF2^{G717R/G717R}$ embryos. Tissue lysates from $eEF2^{+/+}$, $eEF2^{+/G717R}$, and $eEF2^{G717R/G717R}$ embryos were incubated with DT for 30 min in the presence of biotin-NAD. The transfer of biotin-ADP ribose to eEF2 was detected by Western blotting using a streptavidin conjugate. The relative amounts of the eEF2 species that could be ADP ribosylated (after normalization to total eEF2) from $eEF2^{+/+}$ and $eEF2^{+/G717R}$ tissues are indicated under the lanes. (*H*) Body weight trends of male $eEF2^{+/+}$ ($n = 5 \sim 12$ for each time point), $eEF2^{+/G717R}$ ($n = 5 \sim 16$ for each time point), and $eEF2^{G717R/G717R}$ ($n = 4$) mice.

1*H*). Moreover, surviving female and male *eEF2*$^{G717R/G717R}$ mice were fertile and gave normal litter sizes when mated with WT mice.

WT eEF2 as Well as eEF2 G717R Are Less Reactive to the Anti-eEF2 Antibody than the eEF2 from *OVCA1*$^{-/-}$ MEFs. We previously showed that differences in net charge of eEF2 resulting from diphthamide deficiency and ADP ribosylation of WT eEF2 can be detected by changes in electrophoretic mobility on native gels (Fig. S1) (10, 18). We also found that both nonnative forms of eEF2, i.e., the diphthamide-deficient eEF2 from Dph1-, Dph2-, Dph3-, and Dph5-null mutant CHO cells and the ADP-ribosylated eEF2 from WT CHO cells, were more reactive in Western blots with an anti-eEF2 antibody directed to a peptide sequence at the carboxyl terminus of eEF2 (amino acids 741–858) (please refer to figure 3D in ref. 10 and figure 3C in ref. 18). This was observed with blots from native but not SDS gels, indicating that the greater reactivity was due to a conformational difference and that diphthamide may play a role in maintaining the integrity of the eEF2 structure, at least in the C-terminal domains of the molecule. In this study, as expected, the eEF2 from *OVCA1*$^{-/-}$ MEFs migrated on native gels at a rate intermediate between the non–ADP-ribosylated and ADP-ribosylated eEF2 from the WT cells (Fig. 2*A*), indicating the lack of diphthamide, consistent with the expected net charge changes on the diphthamide side chain of the various species at neutral pH (Fig. S1 and Table S3): 0 for His^{715} precursor, "intermediate," and diphthine; +1 for diphthamide; and −1 for ADP ribosyldiphthamide. As observed in CHO cell studies, the eEF2 from *OVCA1*$^{-/-}$ MEFs was more reactive than the WT eEF2 to the anti-eEF2 C terminus antibody on native gels but not on SDS gels (Fig. 2*B*). Notably, the eEF2 G717R from *eEF2*$^{G717R/G717R}$ MEFs retained the electrophoretic mobility property of WT eEF2 on native gels (Fig. 2*B*), apparently because the loss of the positively charged diphthamide modification is compensated by the mutation from neutral Gly^{717} to positively charged Arg^{717} (Table S3). Intriguingly, the eEF2 G717R displayed the same low reactivity to the anti-eEF2 antibody as the WT eEF2 (Fig. 2*A* and Table S3), indicating that the mutated eEF2 more closely resembled the native eEF2 rather than the eEF2 from the *OVCA1*$^{-/-}$ MEFs in conformation. These results suggest that the Gly^{717}-to-Arg mutation may partially correct an unfavorable change in eEF2 conformation resulting from the loss of the diphthamide modification, providing a possible explanation for the data presented above showing that *eEF2*$^{G717R/G717R}$ mice have milder phenotypes than *OVCA1*$^{-/-}$ mice.

In these analyses, whereas the eEF2 in *OVCA1*$^{+/+}$ and *OVCA1*$^{+/-}$ MEFs was efficiently ADP ribosylated when the cells were treated with PA + FP59 for 2 h, the eEF2 in *OVCA1*$^{-/-}$ MEFs was completely resistant (Fig. 2*A*), demonstrating the essential function of mouse OVCA1 in diphthamide biosynthesis. Interestingly, whereas the eEF2 in *eEF2*$^{+/G717R}$ cells was ADP ribosylated to a lesser extent compared with that in *eEF2*$^{+/+}$ cells, ADP ribosylation of the eEF2 G717R from *eEF2*$^{G717R/G717R}$ cells could hardly be detected (Fig. 2*B*).

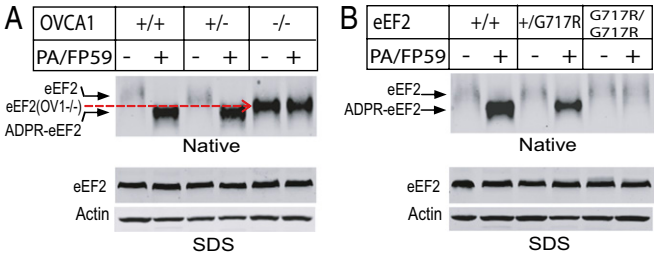

Fig. 2. ADP ribosylation and reactivity of various species of eEF2 to anti-eEF2 carboxyl terminus antibody (*A* and *B*). Cell lysates from *OVCA1*$^{+/+}$, *OVCA1*$^{+/-}$, and *OVCA1*$^{-/-}$ MEFs (*A*) and from *eEF2*$^{+/+}$, *eEF2*$^{+/G717R}$, and *eEF2*$^{G717R/G717R}$ MEFs (*B*) that had been incubated with or without PA and FP59 (1,000 ng/mL and 500 ng/mL, respectively) for 2 h were separated by native PAGE or SDS/PAGE and analyzed by Western blotting using an anti-eEF2 carboxyl terminus antibody (sc-25634; Santa Cruz Biotechnology).

eEF2 G717R but Not the eEF2 from *OVCA1*$^{-/-}$ Cells Retains Full Activity in Polypeptide Elongation. To test the hypothesis that eEF2 G717R may have a higher activity than the eEF2 from *OVCA1*$^{-/-}$ cells, we evaluated protein synthesis rates in *eEF2*$^{G717R/G717R}$ and *OVCA1*$^{-/-}$ MEFs using [^{3}H]-Leu incorporation assays. Remarkably, no differences in protein synthesis rates were observed between *eEF2*$^{+/+}$, *eEF2*$^{+/G717R}$, and *eEF2*$^{G717R/G717R}$ MEFs, demonstrating that eEF2 G717R retains full activity in polypeptide elongation during protein synthesis (Fig. 3*A*). In contrast, the protein synthesis rate in *OVCA1*$^{-/-}$ MEFs was 20% lower than that in the *OVCA*$^{+/+}$ and *OVCA1*$^{+/-}$ MEFs (P = 0.0003, Fig. 3*B*). As a consequence, whereas *eEF2*$^{G717R/G717R}$ MEFs retained the same growth rate as the *eEF2*$^{+/+}$ and *EF2*$^{+/G717R}$ MEFs (Fig. 3*C*), the growth rate of the *OVCA1*$^{-/-}$ MEFs was significantly compromised compared with *OVCA1*$^{+/+}$ and *OVCA1*$^{+/-}$ cells (Fig. 3*D*), agreeing well with the results described previously (20).

We also treated these MEFs with PA + FP59 for 2 h before [^{3}H]-Leu addition. As expected, the protein synthesis rates in WT and *OVCA1*$^{+/-}$ MEFs were completely inhibited and those in *OVCA1*$^{-/-}$ MEFs were not affected (Fig. 3*B*). However, the protein synthesis rate in *eEF2*$^{+/G717R}$ MEFs was decreased only 20–30% (vs. the 50% predicted decrease in the pool of functional eEF2), indicating that eEF2 was only partially rate limiting for protein synthesis. Notably, the protein synthesis rate in *eEF2*$^{G717R/G717R}$ MEFs was also slightly but significantly decreased (10–15%, $P < 0.01$) (Fig. 3*A*), suggesting that the mutated eEF2 retains a residual ADP ribose acceptor activity.

***eEF2*$^{+/G717R}$ and *eEF2*$^{G717R/G717R}$ Cells and Mice Are Resistant to PA + FP59.** We further evaluated the effect of the eEF2 G717R mutation on the sensitivity of cells to PA + FP59. Whereas WT MEFs were highly sensitive to the toxin, the *eEF2*$^{G717R/G717R}$ and *eEF2*$^{+/G717R}$ MEFs were highly resistant to the toxin during 48-h toxin incubations (Fig. S2*A*). However, when these cells were treated with PA + FP59 for extended times, the *eEF2*$^{+/G717R}$ MEFs were completely killed by 120 h, whereas the densities of *eEF2*$^{G717R/G717R}$ MEFs were only modestly (30%) compromised even after 200 h (Fig. S2*B*), consistent with other evidence that the eEF2 G717R activity in protein synthesis can be modestly affected by ADP ribosylation (Fig. 3*A*). In contrast, the *OVCA1*$^{-/-}$ MEFs were completely resistant to PA + FP59 treatment (Fig. S2*C*).

To further evaluate the effects of the eEF2 G717R mutation on the action of the ADP-ribosylating toxins in whole organisms, we challenged WT, *eEF2*$^{+/G717R}$, and *eEF2*$^{G717R/G717R}$ mice with PA + FP59 intraperitoneally. We found that the WT mice were very sensitive to the toxin; all of the mice succumbed to one dose of 5 μg PA + 5 μg FP59 within 1 d (Fig. S2*D*). Native gel analyses of the liver and kidney tissues from moribund challenged WT mice revealed that most of the eEF2 in the tissue lysates existed as the ADP-ribosylated form (Fig. S2*E*). In contrast, both *eEF2*$^{+/G717R}$ (with 50% of total eEF2 lacking diphthamide) and *eEF2*$^{G717R/G717R}$ (all eEF2 lacking diphthamide) mice were resistant to the toxin challenge (Fig. S2*D*). All of the *eEF2*$^{+/G717R}$ mice survived one dose of PA + FP59 challenge and 60% of the *eEF2*$^{+/G717R}$ and all of the *eEF2*$^{G717R/G717R}$ mice survived as many as eight doses of the toxin injection (Fig S2*D*). These results demonstrated that diphthamide deficiency conferred resistance to the ADP-ribosylating toxin at the whole organism level and that the mice could survive even when 50% of the eEF2 were inactivated for as long as 1–2 wk. We also challenged *OVCA1*$^{+/-}$ mice and found that the heterozygous mice were of similar susceptibility as WT mice to PA + FP59 (Fig. S2*F*). These results and the data shown in Fig. 3*B* demonstrate that a single allele of WT OVCA1 is sufficient for diphthamide biosynthesis.

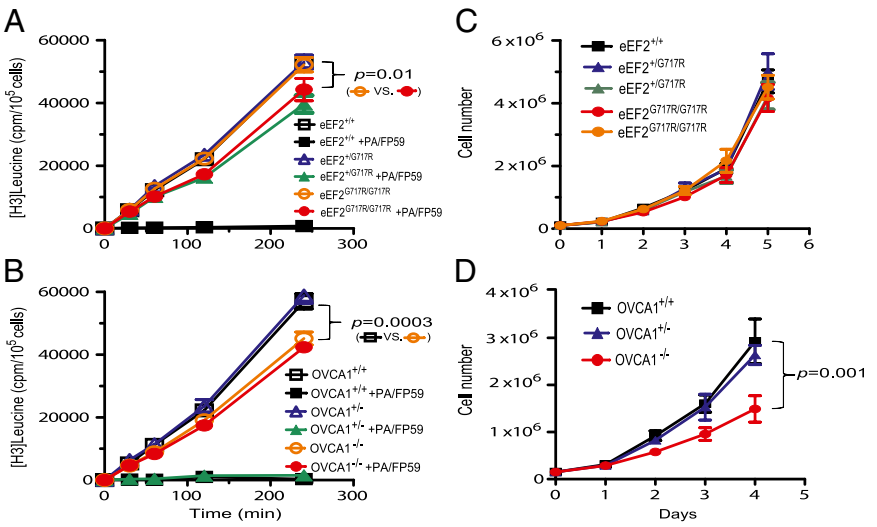

Fig. 3. The eEF2 G717R but not the eEF2 from *OVCA1*$^{-/-}$ cells retains full activity in polypeptide elongation (*A* and *B*). MEF protein synthesis analyses. MEFs with *eEF2*$^{+/+}$, *eEF2*$^{+/G717R}$, or *eEF2*$^{G717R/G717R}$ genotypes (*A*) and with *OVCA1*$^{+/+}$, *OVCA1*$^{+/-}$, or *OVCA1*$^{-/-}$ genotypes (*B*) incubated with or without PA and FP59 (1,000 ng/mL and 500 ng/mL, respectively) for 2 h were incubated with [H^3]-leucine–containing medium for various times as indicated. The incorporated [H^3]-leucine was quantitated by a β-scintillation counter as scintillation cpm. (*C* and *D*) MEF growth rate analyses. MEFs with *eEF2*$^{+/+}$, *eEF2*$^{+/G717R}$, or *eEF2*$^{G717R/G717R}$ genotypes (in *C*, seeded at 1×10^5 cells per well in 6-well plates) and MEFs with *OVCA1*$^{+/+}$, *OVCA1*$^{+/-}$, or *OVCA1*$^{-/-}$ genotypes (in *D*, seeded at 1.5×10^5 cells per well) were allowed to grow and were counted at times as indicated.

Lack of Diphthamide Increases the Error Rate Due to −1 Frameshifts in Protein Synthesis. A recent study on yeast eEF2 mutants provided evidence that diphthamide plays a role in maintaining fidelity in protein translation on ribosomes by preventing −1 frameshifting (29). To investigate whether a similar effect occurs in mouse cells, we transfected the −1 frameshift reporter plasmid pDual-HIV(−1) into *eEF2*$^{G717R/G717R}$, *OVCA1*$^{-/-}$, and WT MEFs. This plasmid encodes a fusion protein of renilla luciferase and firefly luciferase with the downstream firefly luciferase in the −1 frame (Fig. 4*A*) (30). Thus, the renilla and firefly luciferase fusion protein is translated only when −1 frameshifting occurs. Otherwise, a renilla luciferase with a short C-terminal extension is expressed. The ratio of the activities of the two luciferases provides a measure of the extent of frameshifting. Significantly, the −1 frameshift in translation was increased in both *eEF2*$^{G717R/G717R}$ and *OVCA1*$^{-/-}$ MEFs (Fig. 4*B*). Thus, the eEF2 with the G717R substitution does not regain WT translational fidelity, unlike what was seen in the phenotypes examined above. To determine whether diphthamide deficiency caused by inactivation of other Dph genes also increases the −1 frameshift error rate, we transfected pDual-HIV(−1) into a panel of CHO mutants having the Dph1, Dph2, and Dph5 mutations. All these Dph gene mutant cells showed increased −1 frameshift in translation (Fig. 4*C*). Importantly, the −1 frameshift errors could be corrected in ΔDph2 and ΔDph5 cells when plasmids encoding the respective proteins were transfected into these cells (Fig. 4*C*). Taken together, these results show that the diphthamide modification is essential for eEF2 to ensure fidelity in translation. Interestingly, although the Gly717-to-Arg mutation in eEF2 can partially compensate for diphthamide loss in the peptide elongation process, this compensation does not extend to rescue of the −1 frameshifting defect.

Diphthamide Modification on eEF2 Is Crucial in Mouse Development. If the only function of OVCA1 is its role in diphthamide modification of eEF2 and the embryonic lethality of OVCA1-null mice is due to the defect in diphthamide modification, we predicted that doubly mutated mice with an *eEF2*$^{G717R/G717R}$/*OVCA1*$^{-/-}$ genotype would display the milder phenotypes of the *eEF2*$^{G717R/G717R}$ mice. This would follow because the eEF2 substrate for the Dph1 enzyme is missing in *eEF2*$^{G717R/G717R}$ mice, so the absence of Dph1 is silent. This hypothesis was tested by crossing *OVCA1*$^{+/-}$ and *eEF2*$^{G717R/G717R}$ mice, and subsequently breeding *OVCA*$^{+/-}$/*eEF2*$^{G717R/G717R}$ mice to see whether a *eEF2*$^{G717R/G717R}$ background can rescue *OVCA1*$^{-/-}$ mice, which would otherwise be embryonic lethal (Table S2 and ref. 20). Interestingly, whereas only a small fraction of mice with *eEF2*$^{G717R/G717R}$ background could survive to adulthood, ~30% of these mice (three of eight vs. the projected 25%) also possessed OVCA1 deficiency (e.g., *eEF2*$^{G717R/G717R}$/*OVCA1*$^{-/-}$), demonstrating that *eEF2*$^{G717R/G717R}$ can indeed rescue the more severe phenotypes of OVCA1 deficiency. This suggests that the diphthamide deficiency was the cause of embryonic lethality in OVCA1-null mice. Furthermore, similar to *eEF2*$^{G717R/G717R}$ mice, the *eEF2*$^{G717R/G717R}$/*OVCA1*$^{-/-}$ mice that survived to adulthood were fertile when bred with WT mice.

Discussion

The diphthamide modification on eEF2 that is carried out by a set of highly conserved proteins represents one of the most complicated posttranslational biosynthesis pathways, indicating that this unique modification may play an essential role in normal physiology. However, the diphthamide modification is not essential for yeast and mammalian cell survival, suggesting that the modification may only be essential at whole organism levels. Targeted inactivation of Dph genes in mice has provided useful information about the role of diphthamide in normal physiology; however, these genes may also affect other biological processes. Here, we have generated eEF2 G717R mutant mice, in which the first step of diphthamide biosynthesis on His^{715} is prevented.

We previously showed that the diphthamide-deficient eEF2 from Dph gene mutant CHO cells are much more reactive than eEF2 from WT cells to a particular anti-eEF2 C terminus antibody, and this reaction occurs only under nondenaturing conditions (10, 18). The antibody (sc-25634; Santa Cruz Biotechnology) was generated using eEF2 amino acids 741–858 as the immunogenic peptide, and thus do not directly interact with the diphthamide-containing domain (domain IV) of eEF2. Therefore, the antibody reactivity results indicate that the diphthamide-deficient eEF2 may have a different conformation, with more exposed C-terminal epitopes, suggesting that diphthamide contributes to maintaining the conformational integrity of eEF2. Here we found that the eEF2 from MEFs isolated from *OVCA1*$^{-/-}$ mice was also more reactive to the anti-eEF2 antibody. Through analyses of the MEFs isolated from *eEF2*$^{G717R/G717R}$, we found that the Gly^{717}-to-Arg mutation (gain of +1 charge) partially reverses the eEF2 functional loss resulting from diphthamide (+1 charge) deficiency. This is likely due to compensation for the net charge change to eEF2, which may be important for the conformational integrity of eEF2. Indeed, the eEF2 G717R from *eEF2*$^{G717R/G717R}$ MEFs has a low reactivity to the anti-eEF2 antibody, like that of the WT eEF2.

Consistent with the view that the G717R eEF2 is more like WT eEF2, we found it had full activity in polypeptide elongation in protein synthesis and the *eEF2*$^{G717R/G717R}$ MEFs had normal

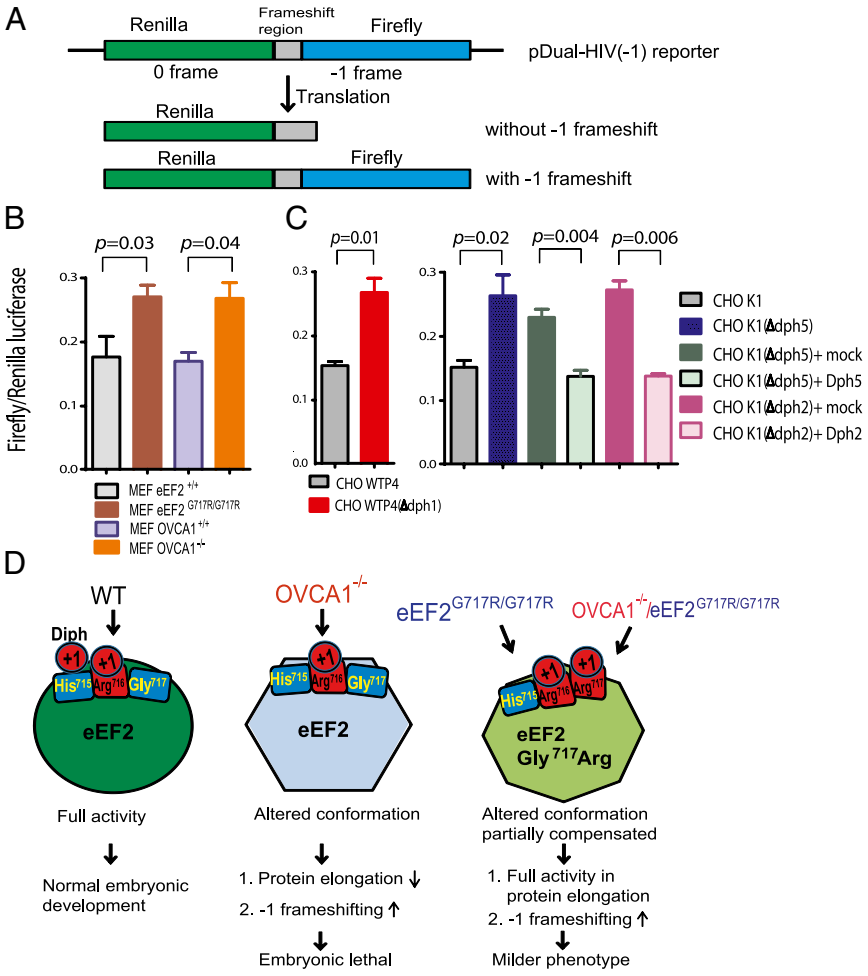

Fig. 4. The lack of diphthamide increases the rate of −1 frameshifting in protein synthesis (*A*). Schematic representation of the −1 frameshifting reporter system. pDual-HIV(−1) encodes a fusion protein of renilla luciferase and firefly luciferase with firefly luciferase in −1 frame. Thus, a fusion protein containing the firefly luciferase is translated only when a −1 frameshift occurs. (*B*) MEFs isolated from *eEF2*$^{+/+}$, *eEF2*$^{G717R/G717R}$, *OVCA1*$^{+/+}$, and *OVCA1*$^{-/-}$ mice were transfected with the reporter plasmid pDual-HIV(−1). Ratios of firefly and renilla luciferase activities were determined 48 h after transfection. (*C*) CHO cells with various Dph gene deletions were analyzed as in *B*. CHO WTP4 is the parental line of Dph1-deficient mutant CHO WTP4(Δdph1). CHO K1 is the parental line of the other CHO mutant cells. The results shown are representative of three individual experiments with similar results. (*D*) Graphical summary of the physiological functions of the diphthamide modification on eEF2.

growth rates. These results are in contrast to those obtained from analyses of *OVCA1*$^{-/-}$ MEFs, which showed significant decreases in both protein synthesis and cell growth rates. Furthermore, the *eEF2*$^{G717R/G717R}$ mice displayed less severe phenotypes in development than did *OVCA1*$^{-/-}$ mice. In contrast to *OVCA1*$^{-/-}$ mice, which have 100% lethality in development, a small fraction of the progeny of *eEF2*$^{+/G717R}$ intercrosses (1.3% of the total compared with the 25% expected to be homozygous *eEF2*$^{G717R/G717R}$ mice) survived to adulthood. Interestingly, for the *eEF2*$^{G717R/G717R}$ mice that survived to adulthood, both the female and male *eEF2*$^{G717R/G717R}$ mice were fertile and gave normal size of litters when mated with WT mice.

Even though the Gly^{717}-to-Arg mutation partially compensated for the defect in function caused by diphthamide deficiency, the *eEF2*$^{G717R/G717R}$ mice still demonstrated a delay in embryonic development so that most died at birth. These results indicate that the *eEF2*$^{G717R/G717R}$ mice are defective in other aspects of diphthamide function critical to embryonic development. We found that diphthamide deficiency on eEF2 does not affect its ability to be regulated via phosphorylation/dephosphorylation on Thr^{56} residue in response to changes in cytosolic amino acids pools (*SI Results* and Fig. S3). A recent study on yeast eEF2 mutants provided evidence that diphthamide plays a role in maintaining the fidelity in protein translation on ribosomes (29). The yeast mutant strains lacking diphthamide modification enzymes show modestly increased −1 frameshifting, an effect not due to a reduction in protein synthesis, ribosome binding, or GTP hydrolysis (29). Remarkably, here we found not only *OVCA1*$^{-/-}$ MEFs but also *eEF2*$^{G717R/G717R}$ MEFs showed significant increases in −1 frameshifting during translation compared with WT cells. Importantly, we further showed that diphthamide deficiency caused by inactivation of other Dph genes in CHO cells also increased −1 frameshifting, and that this could be corrected by complementation with the corresponding Dph-encoding plasmids. These results strongly argue that the diphthamide modification is essential for eEF2 to ensure fidelity in translation. Although the Gly^{717}-to-Arg mutation partially compensated for the loss of diphthamide function as discussed above, the mutation did not rescue the functional loss involving translational frameshifting. One possibility is that the Gly^{717}-to-Arg mutation only partially compensates the altered eEF2 conformation and this is sufficient to rescue the loss in peptide elongation but not in preventing −1 frameshifting. Thus, −1 frameshifting prevention may require a unique conformation that the diphthamide induces in eEF2. A loss in translational fidelity would be expected to lead to the production of damaged and truncated proteins. We hypothesize that the precisely programmed embryonic development process is uniquely sensitive to errors in translation and is less able to tolerate this stress, resulting in death of most of the mice at birth. However, following birth, WT as well as *eEF2*$^{G717R/G717R}$ mice may acquire mechanisms to better deal with damaged proteins.

OVCA1 has been identified as a tumor suppressor (20), suggesting that OVCA1 may also be involved in other biological processes. If the embryonic lethality phenotype of *OVCA1*$^{-/-}$ mice was due to functional loss in diphthamide biosynthesis, we predicted that an *eEF2*$^{G717R/G717R}$ background would rescue the more severe *OVCA1*$^{-/-}$ phenotype, because *eEF2*$^{G717R/G717R}$/*OVCA1*$^{-/-}$ mice contain the more active eEF2 G717R. We found this to be the case; some of the *OVCA1*$^{-/-}$ mice with the *eEF2*$^{G717R/G717R}$ genotype survived to adulthood and were

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fertile when breeding with WT mice. We conclude that the phenotypes of the OVCA1/Dph1 mutant mice result from the loss of diphthamide and that there is no reason to invoke additional, pleiotropic functions for Dph1, with the proviso that the mechanism for the reported tumor suppressor activity of OVCA1 remains unexplained.

In summary, we provide evidence that the unique diphthamide modification is crucial for the full activity of eEF2 in polypeptide elongation and maintenance of translational fidelity (Fig. 4*D*). The eEF2 Gly^{717}-to-Arg mutation maintains eEF2 activity in peptide elongation but does not prevent the loss in fidelity that occurs in the absence of diphthamide. The full activity of eEF2 conferred by diphthamide modification is essential for mouse embryonic development.

Materials and Methods

MEF Cell Growth and Protein Synthesis Assays. MEFs cultured in 96-well plates were treated with or without of PA (1 μg/mL) + FP59 (0.5 μg/mL) for 2 h, or the cells were starved for 1 h in PBS. Then the cells were incubated with medium containing $[^3H]$-leucine (PerkinElmer) (1 μCi per well) for various lengths of time, as indicated. The cells were then harvested and transferred onto protein-binding glass-fiber filters (Pharmacia Wallac) and counted in a β-plate (Pharmacia Wallac) scintillation counter as scintillation count per minute (cpm). Protein synthesis rates were expressed as cpm/10^5 cells.

For cell growth rate analyses, MEFs with $eEF2^{+/+}$, $eEF2^{+/G717R}$, or $eEF2^{G717R/G717R}$ genotype were seeded at 1×10^5 cells per well in 6-well plates and MEFs with $OVCA1^{+/+}$, $OVCA1^{+/-}$, or $OVCA1^{-/-}$ genotype were seeded at 1.5×10^5 cells per well in 6-well plates. The cells were supplied with fresh medium every other day and were counted at times as indicated.

ADP Ribosylation Using Biotin-Labeled NAD. The assay for ADP ribosylation of eEF2 in tissue extracts was performed using biotin-NAD^+. The transfer of biotin-ADP ribose from biotin-NAD^+ to eEF2 in the presence of DT was detected by Western blotting using a streptavidin conjugate as described previously (28). In brief, embryo tissues or adult mouse tissues were lysed in modified RIPA buffer containing protease inhibitors and lysates (5 μL containing 50 μg protein) were mixed with 100 ng DT in ADP-ribosylation buffer (20 mM Tris-HCl, pH 7.4; 1 mM EDTA; 50 mM DTT) with 5 μM 6-biotin-17–NAD (Trevigen) followed by incubation at 25 °C for 30 min. Samples were then mixed with SDS sample buffer, boiled for 5 min, and run on 4–25% SDS/PAGE gels. The proteins were transferred to nitrocellulose membranes using the iBlot system (Invitrogen) and Western blotting was performed using a streptavidin-IR conjugate (Rockland Immunochemicals) and scanned on an Odyssey Infrared Imager (LICOR Biosciences).

Translational Frameshifting Analysis. A total of 1×10^5 MEFs or CHO cells with various genotypes were seeded in 12-well plates overnight and transiently transfected with 2 μg pDual-HIV(−1) using Turbofect (Fermentas) following the manufacturer's instruction. pDual-HIV(−1) encodes a fusion protein of renilla luciferase and firefly luciferase with firefly luciferase in −1 frame (Fig. 4*A*) (30). A frameshift fragment containing a slippery sequence and a stem loop originating from HIV was introduced between the two coding sequences. After a 48-h incubation, firefly and renilla luciferase activities were detected by using the Dual-Glo Luciferase Assay system (Promega) according to the manufacturer's instructions. Firefly luciferase activity, resulting from −1 frameshifting during translation, was detected first in the cell lysates using a Victor 3V plate reader (Perkin-Elmer). Subsequently, firefly luciferase was quenched and renilla luciferase activity was measured in a new buffer system. All measurements were performed in triplicates. *P* values were determined by using *t* test with GraphPad Prism 5 software.

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