Mice Lacking Three Loci Encoding 14 Glutathione Transferase Genes: A Novel Tool for Assigning Function to the GSTP, GSTM, and GSTT Families

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ABSTRACT

Glutathione S-transferases (GSTs) form a superfamily defined by their ability to catalyze the conjugation of glutathione with electrophilic substrates. These enzymes are proposed to play a critical role in protection of cellular components from damage mediated by reactive metabolites. Twenty-two cytosolic GSTs, grouped into seven families, are recognized in mice. This complexity hinders the assignment of function to a subset or family of these genes. We report generation of a mouse line in which the locus encoding three GST gene families is deleted. This includes the four Gst genes spanning 65 kb on chromosome 10 and the seven Gstm genes found on a 150 kb segment of DNA chromosome 3. In addition, we delete two Gstp genes on chromosome 19 as well as a third related gene located 15 kb telomeric to Gstp1 and Gstp2, which we identify as a potential new member of this gene family. We show that, despite the loss of up to 75% of total GST activity in some tissues from these animals, the mice are healthy and fertile, with normal life expectancy. The normal development and health of these animals make them an appropriate model for defining the role of these families in redox homeostasis and metabolism of drugs and environmental pollutants.

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ABBREVIATIONS: CDNB, 1-choloro-2,4-dinitrobenzene; chr, chromosome; DCNB, 1,2-dichloro-4 nitrobenzene; EPNP, 1,2-epoxy-3-(4-nitrophenoxo)propane; ES, embryonic stem; G418, Geneticin; Gst, glutathione S-transferase; kb, kilo base pair(s); LPS, lipopolysaccharide; PCR, polymerase chain reaction; qPCR, quantitative PCR.
detoxification has not been established, a central role for these enzymes in this process is suggested by their unique capacity for conjugation of a tremendous variety of reactive intermediates. As Phase II enzymes, the GSTs conjugate glutathione to xenobiotic electrophiles that are often produced by cytochrome P450-mediated reactions, increasing solubility and thereby facilitating export from cells or enzymatic degradation and subsequent secretion as mercapturic acid.

Mice lacking individual GST genes have provided some functional insight into the role of these enzymes in drug metabolism and risk from environmental carcinogens, especially in the case of GSTP (Henderson et al., 2000; Conklin et al., 2009). However, generating a panel of mice lacking each of the 22 cytosolic mouse GSTs to assess their role in development and normal physiology as well as biotransformation of drugs and xenobiotics would be an arduous undertaking. In addition, the documented variation between the human and mouse isoforms of these genes would call into question the relevance to human health of any experimental findings regarding the function of these GSTs.

As a first step in addressing these obstacles, we have generated mouse lines in which all members of the GSTT, GSTP, or GSTM family, respectively, are completely deleted. Each of these lines was generated from a corresponding embryonic stem (ES) cell line in which all members of a given GST family were excised in a single recombination event. Furthermore, we have interbred the three mouse lines carrying the individual GST family deletions to generate mice lacking these three gene families.

Materials and Methods

Generation of Mouse Lines. Replacement type targeting vectors were constructed from the following 129 derived bacterial artificial chromosomes: Gstp: bMQ145B6, Gsst: bMQ379m08, Gstm: bMQ403h10, and bMQ75m22. Gstm1: bMQ386g24. The region of the mouse genome to be deleted was replaced with a phosphoglycerate kinase-driven neomycin resistance gene. 129S6-derived ES cells were cultured using standard methods, and DNA was introduced by electroporation (Mohn and Koller, 1995). Cells were selected in G418 (Geneticin) and were evaluated by polymerase chain reaction (PCR) and Southern blot analysis using methods previously detailed elsewhere (Koller et al., 1991). Correctly targeted cells were introduced into C57BL/6 blastocysts, which were electroporated (Mohn and Koller, 1995). Cells were selected in G418 (Geneticin) and were evaluated by polymerase chain reaction (PCR) and Southern blot analysis using methods previously detailed elsewhere (Koller et al., 1991). Correctly targeted cells were introduced into C57BL/6 blastocysts, which were returned to B6D2F1/J foster mothers to complete their development. All mice were maintained in specific pathogen-free housing in ventilated caging. The 129S6 mice were purchased from Taconic (Hudson, NY) and bred to chimeras to maintain the deleted loci on this genetic background. Heterozygous animals were intercrossed to obtain mice homozygous for the deletion as well as 129S6 mice. These animals were used to establish mutant breeding colonies and an in-house intercross to obtain mice lacking all three gene families. Each deletion removes all coding sequences as well as regulatory elements that might modulate expression of genes introduced into the loci in the future (Fig. 1). By performing assays of GST function in the mouse line carrying a deletion, each carrying a deletion of one GST family, these lines were in turn interbred to produce the final line lacking all three gene families. Each deletion removes all coding sequences as well as regulatory elements that might modulate expression of genes introduced into the loci in the future (Fig. 1). By performing assays of GST function in the mouse line carrying three deletions, it is possible to determine relatively quickly whether any of the members of these families contributes to the function being assessed. Once a functional deficit is identified in this mouse line, it should be possible to identify the cause of the deficit more narrowly by repeating the assay in each of the lines lacking a single GST family. Because the structures of the deleted loci that we have created are amenable to restoring function by insertion of single or multiple GST genes, it should be possible in the future to engineer mouse lines for pinpointing the GSTs responsible for particular functions. In addition, restoration of function using human GST genes should provide a system for performing functional assays that are more relevant to human physiology.

Results

General Strategy. To create a more streamlined system for the analysis of GST function, we have generated a mouse line lacking all members of the Gstm, Gsst, and Gstp gene families. Because the members of each of these families form a cluster on a different mouse chromosome, each family was deleted separately in mouse ES cells by a single targeted recombination event. The ES cell lines carrying the deleted GST loci were used to generate three mouse lines, each carrying a deletion of one GST gene family. These lines were in turn interbred to produce the final line lacking all three gene families. Each deletion removes all coding sequences as well as regulatory elements that might modulate expression of genes introduced into the loci in the future (Fig. 1). By performing assays of GST function in the mouse line carrying all three deletions, it is possible to determine relatively quickly whether any of the members of these families contributes to the function being assessed. Once a functional deficit is identified in this mouse line, it should be possible to identify the cause of the deficit more narrowly by repeating the assay in each of the lines lacking a single GST family. Because the structures of the deleted loci that we have created are amenable to restoring function by insertion of single or multiple GST genes, it should be possible in the future to engineer mouse lines for pinpointing the GSTs responsible for particular functions. In addition, restoration of function using human GST genes should provide a system for performing functional assays that are more relevant to human physiology.

Generation of Mice Lacking the Gsst Locus. The two mouse Gsst genes, Gsst1 and Gsst2, which lie in close proximity (~2.4 kb) to one another on chr 19 between Cdh5 and Ndufv1, have been characterized. However, during the design of a vector to remove these two genes and the upstream regulatory elements, we identified an additional GST-like gene in this region, designated BC021614 (Fig. 1A). Sequence analysis shows that this gene is most closely related to the mouse and human Gsst genes. A blast search of the human genome with the sequences from BC021614 identifies GSTP1 as the only human ortholog. Comparison of the protein structure encoded by this sequence to mouse and human GSTP further supports the contention that BC021614 represents a previously unrecognized mouse Gsst gene.
Fig. 1. Schematic for generation of mice lacking the mouse Gstp, Gstt, and Gstm loci. All loci are shown with the proximal (centromere) on the left and distal (telomere) to the right. Genes are shown as cylinders, and the 5′ to 3′ orientation of each is indicated by a black arrow below. Intergenic DNA is shown as a thin black line. Distances are approximately proportional but not to scale. The region deleted is indicated above each locus. Genes in the deleted region are shown as red cylinders. The restriction fragment and the probe used for initial verification of the targeting event by Southern blot analysis are shown for approximately proportional but not to scale. The region deleted is indicated above each locus. Genes in the deleted region are shown as blue cylinders; genes outside the deleted region are shown as red cylinders. The restriction fragment and the probe used for initial verification of the targeting event by Southern blot analysis are shown for

A Gstp locus

- HindIII
- 11.5 kb
- 40 kb deletion
- probe

Gstp1

Gstp2

Gstp3

Calcβ2

(BCO21614)

B Gstt locus

- EcoRI
- 65 kb deletion
- 8.7 kb
- probe

Calb1

Ddt

Gstt1

Gstt2

Gstt3

C Gstm locus

- Apal
- 15 kb
- 150 kb deletion
- probe

Eps8l3

M3

M3

M3

M3

M3

Ampd2

Together, our findings suggest that it is likely that BCO21614 represents an additional member of the mouse GST pi family. Although clearly assignment of BCO21614 to the Gstp gene family awaits demonstration that the encoded protein catalyzes the conjugation of glutathione with electrophilic substrates, for simplicity, we refer to this gene as Gstp3 throughout this study. Importantly, this analysis indicates that generation of a mouse lacking all GSTP activity might require removal of this gene, which lies approximately 15 kb telomeric to the Gstp1/2 genes. Thus, a targeting vector was designed that removes ~40 kb of DNA. This includes the three Gstp genes and intergenic DNA as well as ~8.5 kb of DNA upstream of Gstp3 and 7 kb of DNA downstream of Gstp1 that may be involved in regulation of the locus. ES cells in which this targeting construct integrated by homologous recombination were identified by PCR analysis and verified by Southern blot analysis (Supplemental Fig. 1A). Mice homozygous for the deleted locus (Gstp(DΔ)) were generated, and DNA and RNA were analyzed to confirm the loss of the segment of DNA carrying the three genes. DNA was probed with full-length cDNA probes corresponding to Gstp1 and Gstp3. No binding of either probe was observed in DNA obtained from the GstpΔΔ mice (Supplemental Fig. 2A). These cDNA probes were also used to confirm loss of expression of the genes. As expected, a Gstp1/2-specific cDNA probe hybridized to RNA prepared from the duodenum and colon of wild-type mice. The high homology between these two genes (99.3% by Wilbur-Lipman DNA alignment) does not allow generation of a probe that distinguishes between the two transcripts. Gstp3 expression was also detected by Northern blot analysis of total
RNA prepared from the gut by hybridization with a full-length cDNA probe corresponding to Gstp3. Neither probe detected Gstp-derived transcripts in RNA prepared from GstmΔΔ mice (Fig. 3A).

**Generation of Mice Lacking the Gstt Locus.** The theta locus on mouse chr 10 contains four Gstt genes as well as Ddt (d-tophosphate tautomerase) clustered between Cabin1 (calcineurin-binding protein 1) and Mif (macrophage migration inhibitory factor). Ddt is located in close proximity to Gsst3 (Fig. 1B). Because of the possible overlap in the regulatory elements controlling Ddt and Gsst3, we included this gene in the deletion, thereby removing mouse genes between Cabin1 and Mif. Targeted ES cells were identified by PCR and Southern blot and were used to generate a mouse line homozygous for the deleted Gsst locus (GsstΔΔ), (Supplemental Fig. 1B). The correct deletion of the loci was verified by analysis of DNA and RNA from homozygous GsstΔΔ mice. Analysis of DNA with a number of probes unique to the GsstΔΔ locus failed to bind to DNA prepared from these animals (Supplemental Fig. 2B). DNA was prepared from liver and kidney and was analyzed with cDNA probes specific for (Supplemental Fig. 2B). The hybridization pattern of the probe with DNA from two of the eight pups derived from the intercross of Gstm+ΔΔ mice is consistent with the absence of all the Gstm genes in these animals. We also verified the loss of expression of each of the seven genes. Based on our own studies and published reports, we identified tissues with high levels can be observed in wild-type animals (Fig. 3C).

**Generation of Mice Lacking the Entire Gstm Locus.** The mouse Gstm locus spans 150 kb and contains seven Gstm genes (Fig. 1C). Targeted ES cells were identified by PCR and Southern blot analysis (Supplemental Fig. 1C), and these cells were used to generate a GstmΔΔ mouse line. DNA and RNA from these mice were used to verify the deletion of the locus, including all seven Gstm genes. In this case, because of the complexity and size of the deletion, we generated a probe that would allow a comprehensive evaluation of the deletion by Southern blot analysis (Fig. 4). DNA from the homozygous GstmΔΔ mice was digested with a number of enzymes and subjected to Southern blot analysis using a probe corresponding to the region of DNA extending from the middle of exon 3 to just downstream of exon 4 of Gstm6. Genome analysis showed that this fragment of DNA shares sufficient homology to allow detection by Southern blot analysis of eight regions in the Gstm gene cluster, with only minimum homology to four other regions in the mouse genome (Supplemental Table 1). An example of evaluation of such an analysis is shown (Fig. 4).

The hybridization pattern of the probe with DNA from two of the eight pups derived from the intercross of Gstm+ΔΔ mice is consistent with the absence of all the Gstm genes in these animals. We also verified the loss of expression of each of the seven genes. Based on our own studies and published reports, we identified tissues with the highest expression for each of the seven genes. RNA was prepared from these tissues from wild-type mice as well as mice homozygous for the GstmΔΔ locus and was analyzed by quantitative PCR (qPCR). No expression could be detected for any of these genes, even in tissue in which high levels can be observed in wild-type animals (Fig. 3C).

As an additional control for future studies assigning function to various Gstm genes, we generated a mouse line lacking only Gstm1. We chose to generate a line lacking this individual gene because our expression analysis as well as published studies (Knight et al., 2007) indicated that expression of this gene dominates in many tissues, and thus direct comparison of the GstmΔΔ mice and Gstm1−/− mice would be important in future studies assigning function to other members of this large gene family. The 18.6 kb segment of the Gstm locus encoding Gstm1 was deleted, and loss of the locus and lack of expression of Gstm1 were verified by Southern blot and qPCR analysis of homozygous Gstm1−/− animals (Supplemental Fig. 3). The deletion includes the entire gene as well as promoter elements, thus mimicking the deletion event that resulted in the null Gstm1 allele present in humans. We verified that this deletion does not result in loss of expression of the neighboring two genes, particularly Gstm2 and Gstm4 (data not shown).

**Generation of Mice Lacking Gstp, Gstt, and Gsst Genes.** The null allele for each of the three loci was maintained on the 129S6 genetic background by mating the chimeras generated from the three ΔES cell lines directly with 129S6 female mice. This makes it possible to generate coisogenic 129 mice in which the only genetic difference between experimental animals and control animals is the absence of these three loci. All DNA flanking these mutations will be of 129S6 origin. We first generated a mouse line lacking both the Gsst and Gstp loci. This line was then intercrossed with the GstmΔΔ mice to generate the mice lacking all three gene families. These GstmΔΔ/GsstΔ−/−/GstpΔ−/− mice
mice appeared normal and could not be visually distinguished from 129S6 mice. The weight of the three mouse lines did not differ significantly when examined through the first 8 weeks from control 129S6 animals (Supplemental Table 2). Morphologic evaluation of tissues showed no gross abnormalities, including in size of organs, nor did histologic evaluation reveal an overt impact of loss of these 14 genes on development (data not shown). For example, histologic evaluation of liver, lung, and kidneys indicated normal development of these organs, and changes indicating increased cell death and/or inflammation were not observed. Staining with Oil Red revealed no fatty changes to the liver; an increase in fibrosis, evaluated by staining sections with Masson’s trichrome, was not observed in the ΔPMT animals. Changes consistent with ongoing activation of the immune system were not apparent on evaluation of the leukocyte composition of the blood, thymus, spleen, and lymph nodes (Supplemental Table 3). A decrease in the circulating number of B cells was observed; however, the change was small, and normal B cell numbers were observed in both the spleen and nodes. Biochemical analyses showed no difference in hematocrit, red cell, or platelet numbers between ΔPMT mice and 129S6 controls.

Fig. 3. Expression of the Gstp, Gstm, and Gstt genes in the mice homozygous for the deleted loci. (A) RNA was prepared from the duodenum (duo) and colon of two GstpΔ/Δ mice and their controls and was analyzed by Northern blot using the a full-length cDNA probe. The Gstp1 cDNA does not discriminate between Gstp1 and Gstp2 expression because of the high level of homology between these genes (99.3% by Wilbur-Lipman DNA alignment). As expected, a strong signal was observed in the lanes corresponding to wild-type mice but not in the lanes loaded with RNA from GstpΔ/Δ animals. A band is also detected in wild-type RNA from these tissues when analyzed with a full-length cDNA probe corresponding to Gstp3. Although Gstp3 has 71.4% (Lipman-Pearson protein alignment) homology at the protein level with Gstp1/2, this cDNA probe has only 65% similarity index (Wilbur-Lipman DNA alignment) with Gstp1 and Gstp2 and thus will not hybridize to these transcripts in the conditions used in this study. Consistent with a deletion that encompasses this telomeric Gstp gene, this band is absent in lanes corresponding to the GstpΔ/Δ mice. To verify the quality and quantity of the bound RNA, filters were probed with mPges2, a gene that is expressed at moderate levels throughout the intestinal tract. (B) RNA was isolated from liver, kidney, and bone marrow derived macrophages of wild-type and GsttΔ/Δ mice. Northern blot analysis using cDNA probes specific for Gstt1 and Gstt2 under stringent hybridization conditions showed high expression of both genes in these tissues. Gstt3 expression, detected using a full-length cDNA, was also observed in both liver and kidney, although longer exposure of the film was required. No signal was observed using the three Gstt probes in RNA prepared from GsttΔ/Δ mice. Gstt4 is expressed at low levels in most tissues, including bone marrow-derived macrophages. However, we identified robust expression of this gene after treatment of macrophages for 16 hours with LPS using a full-length cDNA probe specific for this gene. No signal was observed in lanes corresponding to RNA isolated from LPS-treated cells derived from GsttΔ/Δ mice. (C) Total RNA was prepared from the indicated tissues of GstmΔ/Δ mice and cohoused sex- and age-matched controls. Expression of the Gstm gene indicated in each of the panels from tissues known to express each family member was determined by qPCR using gene-specific primers obtained from Applied Biosciences. Expression levels were normalized to 18S RNA. Data were analyzed using the comparative C\textsubscript{t} method (ΔC\textsubscript{t}) as described by Applied Biosystems. No signal (ND: not detected) was observed in all cases in samples prepared from mice homozygous for the GstmΔ/Δ locus. Values represent mean of three animals ± S.E.M.
and GST-CDNB activity measured (Fig. 6). A dramatic decrease in activity toward this substrate. Cytoplasmic fractions were prepared from the liver, lung, and kidney of the male and female animals (Supplemental Table 4). The only significant difference observed was a slight decrease in the levels of alkaline phosphatase in animals (Supplemental Table 4). The only significant difference observed was a slight decrease in the levels of alkaline phosphatase in animals. However, although no significant difference in the magnitude of the change in CDNB-GST activity was observed between these two groups, suggesting that the CDNB activity in this family is attributable primarily to GSTM1 (Fig. 6B). The large decrease in GST-CDNB activity cannot be accounted for by loss of the GSTl locus. Consistent with the reported inability of GSTT to metabolize CDNB, no change in CDNB activity was observed in extracts from the GstmΔΔ mice (data not shown). Together our results indicate that the loss of all three loci has dramatic impact on total CDNB activity that cannot be accounted for by the loss of the Gstm1 and GSTP alone.

We also examined activity of cytosolic extracts prepared from the ΔPMT mice toward DCNB as a substrate preferentially metabolized by GSTM (Fig. 7A). A 90% reduction in activity was observed in extracts from the liver, kidney, and lung of ΔPMT mice. Although no change in activity toward this substrate is observed in extracts from the GstmΔΔ mice, a decrease of similar magnitude is observed in the GstromΔΔ mice, assigning the GST-DCNB activity to this family. Evaluation of mice lacking only Gstrom1 assigns all GST-DCNB activity to this family member, as the magnitude of the decrease in GST-DCNB activity in the cytosolic fractions from the Gstrom1−/− animals was not significantly different than the activity of the GstromΔΔ tissues.

The activity toward EPNP is also dramatically reduced in tissues from the ΔPMT mice (Fig. 7B). EPNP is reported to be metabolized primarily by mouse GSTT1. Consistent with this report, comparison of the activity toward this substrate in cytosolic fractions from GstmΔΔ mice and ΔPMT mice assigned all activity to this locus. Similarly, activity toward ethacrynic acid, a substrate for GSTP, was reduced in liver extracts from ΔPMT and GstmΔΔ animals (data not shown).

These experiments using substrates metabolized primarily by GSTT, GSTM, and GSTP demonstrate the facility of using the ΔPMT in combination with the mice lacking individual loci and individual genes to assign the metabolism of compounds for which metabolic pathways have not been established, first generally to these GST families, and then specifically to a family of these enzymes.

**Discussion**

We have successfully generated mice lacking the Gsto, Gstm, and Gspt gene families, as confirmed by analysis of the structure of the DNA from the deleted loci as well as evaluation of the expression of the 14 genes located in these three major Gsto families. In all cases, the loss of expression of the Gsto families is the result of the deletion of the entire locus, including DNA flanking the telomeric and centromeric
boundaries of the gene clusters. Thus, it is not surprising that we detect no expression of any Gstp, Gstt, or Gstm family members in mice homozygous for each of the deleted loci, even when tissue that normally displays the highest level of expression is chosen for study.

When choosing the boundaries for each of the three deletions, the organization and structure of the genes adjacent to the deleted loci were considered. In silico analysis of the regions to be deleted was performed to identify conserved, noncoding sequences of possible regulatory importance as well as transcribed regions of unknown function. Although the deleted Gst gene clusters were flanked in most cases by poorly conserved, intergenic DNA, an exception was the region between Gstt3 and Ddt, the gene encoding D-dopachrome decarboxylase, at the centromeric boundary of the Gst locus. In humans, the DDT gene is located within the GSTT cluster (Coggan et al., 1998). The name of this gene describes its enzymatic activity, conversion of D-dopachrome into 5,6-dihydroxyindole (Odh et al., 1993). However, D-dopachrome is not present in vivo, and more recent studies suggest that DDT, similar to migration inhibitory factor (MIF), binds CD74 (Merk et al., 2011). This activity suggests that, like MIF, DDT may in fact be a cytokine involved in regulation of immune responses. However, because potential regulatory regions of the Gstt3 extend into the Ddt gene, this gene was destroyed during the deletion event, as we decided it was prudent to remove all sequences in this region with the potential to influence expression of GSTT or Gstt genes introduced into the delta Gstt locus in future experiments.

Should the ΔPMT mice display a phenotype that is subsequently attributed to the Gstt locus through study of the GsttΔΔ mice, we can assign the phenotype to one of the human or mouse Gstt genes using mice generated from ΔPMT ES cells reconstituted with various segments of the mouse Gstt or human GSTT locus. A line restored with only the Ddt gene would serve as a control.

The close chromosomal proximity of the Gstp1 and Gstp2 genes in the mouse has facilitated the use of gene targeting in a previous study to generate mice in which a mutation is introduced that disrupts
expression of both genes (Henderson et al., 1998). We noted, however, on examination of this locus, the presence of a gene that, based on its primary structure, appears to represent a third member of the Gstp gene family. There are a number of intriguing features of this gene that we believe warrant its further study. First, the dramatic increase in the expression of Gsp1/2 in male mice observed at puberty (Conforto and Waxman, 2012) is not conserved in Gsp3. Second, although we have observed lower transcript levels of Gsp1/2 in the tissues examined to date, this may be compensated for by the fact that the predicted protein encoded by this gene includes an amino acid substitution that has been shown to increase the stability of human GSTP. Final assignment of this gene to the Gsp family awaits determination of whether the protein encoded by BC021614 (Gsp3) has GST activity.

Evaluation of GST activity in the four mouse lines we have generated toward molecules known to be preferred substrates for members of the Gstp, Gstt, or Gstm family, respectively, demonstrates the utility of these lines in defining the role these GST families play in the metabolism of a particular substrate. Comparison of the results of these evaluations with similar evaluations in mice lacking single genes can subsequently be used to determine the contribution of individual family members to the metabolism of a particular substrate. For example, comparison of the activity of extracts from Gsm1ΔΔ mice and Gsm1−/− mice indicates that Gsm2 through Gsm7 do not contribute significantly to the metabolism of DCNB, even though the expression of Gsm2, Gsm3, Gsm6, and Gsm4 in the liver has been reported (Knight et al., 2007). Interestingly, metabolism of DCNB by human GSTM1 has not been supported in comparative studies using extracts from individuals carrying the null GSTM1 allele (Arakawa et al., 2012). Therefore, this substrate may be highly specific for a single murine Gstm1 gene, calling into question the evolutionary and functional relationship between the mouse and human Gstm isoforms (Board, 2007).

The GspΔΔ mice demonstrated only a small decrease in activity toward CDNB in the liver and kidney, and no change in metabolism of this substrate could be measured in extracts from the lung. This differs in some important aspects from results reported for the Gsp1/2 mice. In an initial study of these animals, Henderson et al. (1998) reported that the activity toward CDNB was reduced in lung cytosolic fractions but not in the fractions prepared from the kidney and liver. However, a later study reported reduced activity toward CDNB in cytosolic extracts prepared from liver, lung, and kidney of the same mouse line, with the most dramatic reduction observed in the liver (Conklin et al., 2009). A possible explanation for these disparate findings is the different genetic background of the GSTP-deficient mice. Our studies were conducted with coisogenic 129S6 animals whereas the initial studies of the Gsp1/2−/− mice were performed with F2 mice carrying 129 and MF1 alleles; the subsequent studies used mice in which the mutation had been introduced into the C57BL/6 background by successive rounds of breeding. Strain differences in mouse liver
GST activity have been previously noted (Egaas et al., 1995). Differences might reflect different environmental conditions, including biologic differences in the microflora colonizing skin, intestinal, and/or nasal passages as well as physical differences such as bedding and diet.

Perhaps the most surprising finding of our study of GST activity present in the cytosolic fractions prepared from the ΔPM'T mice was the robust and easily repeatable decrease in the metabolism of CDNB by cytosolic fractions from prepared organs, particularly the lung. This change could not be assigned to the Gsst genes, as the GstaΔΔ mice showed no change in activity toward CDNB, consistent with other studies. Furthermore, although some of the activity toward CDNB could be attributed to the Gstm1 gene, the contribution from this gene represented a small fraction of the total activity. As discussed earlier, only a small change in activity toward CDNB was observed in the GspΔΔ mice, and this was limited to the liver and kidney. No decrease was seen in the lung, the tissue with the largest decrease in activity toward CDNB in the ΔPM'T animals. Taken together, the decreases in activity observed in the GspΔΔ and GstaΔΔ mice cannot account for the dramatic decrease in the Δ PM'T animals. One possible explanation is that there is a compensatory up-regulation of genes in the GspΔΔ and GstsΔΔ mice. Compensation for loss of a Gst gene was reported for Gstad/P−/− mice, where increased expression of Gsta2, Gsta3, and Gstm1 was observed (Engle et al., 2004). In comparison, we observed no change in Gsta, Gst1, or Gskl expression and only a small increase in mgstl in female ΔPM'T mice. However, we did not examine tissue from the GspΔΔ mice or the GstaΔΔ mice for changes in gene expression. A possible role for compensatory changes in gene expression between the Gstp and the Gstm loci can be tested by the generation of mice lacking only these two loci.

The mice lacking the 14 Gst genes are remarkably healthy and fertile, with no apparent increase in morbidity. Evaluation of blood chemistry from three of the oldest available animals indicated no significant loss of liver or kidney function. The normal development and good health of these animals suggests that these enzymes are not required to maintain redox homeostasis under normal conditions. This interpretation is supported by our failure to detect an increase in the expression of antioxidant enzymes, a signature of oxidative stress. This suggests that the Gstm, Gstp, and Gstt genes function primarily in response to changes in environmental stress factors that are largely consistent or limited in a vivarium. Such an interpretation would be consistent with the significant differences between humans and rodents in the number, substrate specificity, and organization of these gene families, as species differences would reflect the very different environmental pressures driving evolution of these genes in humans and mice. Mice in which the deleted loci are restored with individual mouse genes or, alternatively, with their proposed human orthologs should provide models for pharmacologic and toxicologic studies of the role of these phase II enzymes in the metabolism of drugs and xenobiotics.

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Authorship Contributions

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