Mice lacking three loci encoding fourteen glutathione transferase genes: a novel tool for assigning function to the GSTP, GSTM, and GSTT families

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Mice lacking the GSTP, GSTM, and GSTT families

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ABBREVIATIONS: BAC, bacterial artificial chromosome; CDNB, 1-choloro-2,4-dinitrobenzene; Cyp, cytochrome P450; Chr, chromosome; DCNB,1,2-dichloro-4 nitrobenzene; EDTA, ethylenediaminetetraacetic acid; EPNP, 1,2-epoxy-3-(4-nitrophenoxy)propane; ES, embryonic stem; FACS, fluorescence-activated cell sorting; Gst, glutathione S-transferase; kb, kilo base pair(s); LPS, Lipopolysaccharide; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; RT-PCR, reverse transcription-polymerase chain reaction; qPCR, quantitative PCR.
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 Gstt 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.
Introduction

Glutathione S-transferases (GSTs) catalyze the conjugation of glutathione (GSH) with electrophilic molecules (Hayes et al., 2005). These enzymes facilitate this catalysis through proton extraction from GSH. Various GST isoforms have now been identified in a variety of complex organisms, with over twenty present in the mouse alone. The GST enzymes are generally grouped into one of three classes based on their subcellular localization: membrane-bound microsomal, mitochondrial and cytoplasmic. Membrane bound microsomal GSTs include enzymes such as PTGES in which glutathione transfer plays a critical role in the synthesis of prostaglandins (Trebino et al., 2003). Mammals express a single, evolutionarily divergent mitochondrial GST. It was presumed because of its mitochondrial localization to have a critical role in redox processes. However, mice lacking the gene encoding this mitochondrial GST are relatively healthy, with glomerular changes in males being the only pathology reported (Blackburn et al., 2011). The largest and most diverse group of GSTs encompasses those present in the cytosol. Cytosolic GSTs are further grouped into the following families based on substrate specificity and, more importantly, sequence similarity: alpha (A), mu (M), pi (P), theta (T), sigma (S), zeta (Z), and omega (O). The families vary in size from one to seven genes, and when there are multiple family members, the genes encoding these are linked in loci that suggest expansion form a primordial founding gene. Thus, for example, the seven mouse Gstm genes are located in a single locus on chromosome 1.

Significant progress has been made in understanding the function of some GST families, especially those consisting of a single enzyme. This progress has resulted from extensive biochemical studies as well as from studies using knockout mice in which individual GST genes have been inactivated by homologous recombination (Henderson et al., 1998; Engle et al., 2004;
Lim et al., 2004; Fujimoto et al., 2006; Fujimoto et al., 2007). For example, GSTZ plays an important role in the catabolism of aromatic amino acids, as it catalyzes the isomerization of maleylacetoacetate to fumarylacetoacetate. Generation of mice in which the gene encoding GSTZ1-1 had been inactivated by targeted recombination showed that this enzyme plays an important role in this pathway, as mice lacking this enzyme are very sensitive to any increase in tyrosine or phenylalanine in their diet (Lim et al., 2004). The high conservation between human and mice of the encoded protein (>80%) and the fact that a single GSTZ1 gene is present in both species allows extrapolation of the result from this experimental system to humans.

Unlike the GSTZ gene however, many GSTs belonging to larger families display considerable interspecies variation in family size as well as in the expression pattern and substrate preferences of various family members. A role for the members of the various GST families in protection of the cell against endogenous metabolites has not been ascertained. Although the relative importance of the GST families in 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 Cyp 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 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 BACs: *Gstp*: bMQ415B6, *Gstr*: bMQ379m08, *Gstm*: bMQ403h10 and bMQ75m22, *Gstm1*: bMQ386j24. The region of the mouse genome to be deleted was replaced with a PGK driven neomycin resistance gene. 129S6 derived ES cells were cultured using standard methods, and DNA was introduced by electroporation (Mohn, 1995). Cells were selected in G418 (Geneticin) and evaluated by PCR and Southern blot using previously detailed methods (Koller et al., 1991). Correctly targeted cells were introduced into C57BL/6 blastocysts which were returned to B6D2F1/J foster mothers to complete development. All mice were maintained in specific pathogen free housing in ventilated caging. 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’ 129S6 colony. Mutant mice were age and sex matched at weaning and housed together until used in an experiment. All mice used in these studies were co-isogenic for the locus under study. Mice were genotyped using primer sets described (Supplementary Methods). All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee guidelines of the UNC Chapel Hill.
Expression analysis. Total RNA was isolated from tissue using RNA isolation solvent (RNA-Bee, Tel-Test Inc, Friendswood, TX, USA) according to the manufacturer’s protocol. Northern blot analysis was conducted using a $^{32}$P-labeled probe prepared from the cDNA probes indicated in the figure legend. Reverse transcription of RNA to cDNA for quantitative RT-PCR was performed using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Primers and probes were purchased from Applied Biosystems, Foster City, CA. DNA was amplified with FastStart Universal Probe Master (Roche Applied Science, Indianapolis, IN). All samples were run in duplicate, and relative expression was determined by normalizing samples to 18S RNA ($\Delta$CT). To facilitate use of this highly expressed internal standard, samples were diluted to 10 pg/µl for analysis with this primer set, compared to a dilution of 2.5 ng/µl for all other probe sets. Data were analyzed using the comparative $C_T$ method as described by Applied Biosystems. In some experiments samples from mutant animals were normalized to values obtained from co-housed control animals ($\Delta\Delta$CT).

Hematological and clinical chemistry. Blood samples were collected in EDTA (5 mM) from deeply anesthetized animals by cardiac puncture and analyzed using Heska’s Animal blood counter. Clinical chemistry was carried out on plasma prepared from the blood using an Automatic Chemical Analyzer (Johnson and Johnson’s V350, New Brunswick, NJ).

FACS analysis. Cells from whole blood (white blood cells), spleen, thymus and lymph nodes isolated from $\Delta PMT$ or control mice 6-8 weeks of age were stained with markers specific for T cells (CD3, CD4, CD8), B cells (B220), myeloid cells (CD11, GR-1). Fluorescence-activated cell sorting (FACS) was carried out using CyAn™ ADP Analyzer (Beckman Coulter, Inc., Brea, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).
**Measurement of GST Activities in mouse tissues.** Mice were transcardially perfused with PBS pH 7.4 to remove red blood cells from the tissue. 150 mg of tissue was homogenized in 1 ml of cold buffer (100 mM potassium phosphate pH 7.0, 2 mM EDTA) and centrifuged at 10,000 x g for 15 min at 4 °C to enrich for cytosolic and microsomal GSTs. To further enrich for cytoplasmic GSTs the supernatant was transferred to new tubes and centrifuged at 10,000 x g for 60 min at 4 °C. GST activity in the supernatant was then determined spectrophotometrically as previously described (Habig et al., 1974). Substrates used in this analysis include: Ethacrynic acid (TCI, Portland, OR); 1,2-Epoxy-3-(4-nitrophenoxy) propane (EPNP), (Oakwoods Chemical, West Columbia, SC); 1,2-dichloro-4 nitrobenzene (DCNB) Sigma-Aldrich, St. Louis, MO; 1-chloro-2,4-dinitrobenzene (CDNB) Sigma-Aldrich, St. Louis, MO.

**Statistical Analysis.** The method used for the evaluation of data is detailed in each figure legend.
Results

General strategy. In order to create a more streamlined system for the analysis of GST function, we have generated a mouse line lacking all members of the \textit{Gstm}, \textit{Gstt} and \textit{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 carrying out assays of GST function in the mouse line carrying all three deletions, it should be possible to determine relatively quickly whether any of the members of these families contributes to the function being assayed. 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 carrying out functional assays that are more relevant to human physiology.

Generation of mice lacking the \textit{Gstp} locus. The two mouse \textit{Gstp} genes, \textit{Gstp1} and \textit{Gstp2}, which lie in close proximity (~2.4 kb) to one another on chr 19 between \textit{Cabp2} and \textit{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 *Gstp* genes. A blast search of the human genome with the sequences from *BC021416* 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 *BC021416* represents a previously unrecognized mouse *Gstp* gene.

We aligned the known GSTP protein sequences and examined that extrapolated from the sequence of *BC021416* for the presence of residues conserved between species and/or shown, using a mutagenesis approach, to play a role in enzyme activity (Fig. 2A) (discussed in (Vasieva, 2011)). Tyr 8 is critical for many aspects of GSTP function including folding and structural mobility, interactions with other proteins, substrate binding and catalysis (Oakley et al., 1998; Pettigrew et al., 1999). Trp 39, Lys 45 and Gln 52 contribute to the GSH binding site (Oakley et al., 1998). Cys 48 and Tyr 80 are involved in binding and regulatory activities such as heterodimerization (Ralat et al., 2006). Chemical modification studies of porcine lung pi class GST suggest that Tyr 109 may be located near or at the binding site (Pettigrew et al., 1999), while Tyr 154 has been shown to contribute to the hydrophobic staple motif (Stenberg et al., 2000b). Tyr 50 is a key residue at the dimer interface and has been implicated in the cooperativity of the enzyme (Stenberg et al., 2000a). All of these residues are conserved in mGSTP1, mGSTP2, hGSTP1 and the predicted protein encoded by *BC0221614* except for Tyr 50 that is replaced by Phe in the *BC0221614* protein. Interestingly, this substitution has been shown in human GSTP1 to result in higher thermal stability and catalytic efficiency (Stenberg et al., 2000a). *BC0221614* is an expressed gene, with transcripts identified in a number of tissues including the liver. In contrast to the conservation of functionally important amino acids among
the three mouse GSTP proteins, expression of BC0221614 does not display the well characterized sexual dimorphism of Gstp1 and Gstp2. Unlike the expression of these genes, expression of BC0221614 is similar in the male and female liver in both young and sexually mature animals (Fig. 2B).

Together our findings suggest that it is likely that BC021614 represents an additional member of the mouse GST pi family. Although, clearly assignment of BC0221614 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 requires 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 (Supplemental Figure 1A). Mice homozygous for the deleted locus (GstpΔ/Δ) were generated and DNA and RNA 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 GstpΔ/Δ mice (Supplemental Figure 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 GstpΔ/Δ mice (Fig. 3A).

**Generation of mice lacking the Gsst locus.** The theta locus on mouse chr 10 contains four Gsst
genes as well as Ddt (D-dopachrome 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 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 used to
generate a mouse line homozygous for the deleted Gsst locus (GsstΔ/Δ), (Supplemental Figure
1B). The correct deletion of the locus was verified by analysis of DNA and RNA from
homozygous GsstΔ/Δ mice. Analysis of DNA with a number of probes unique to the 65 kb Gsst
locus failed to bind to DNA prepared from these animals (Supplemental Figure 2B). RNA was
prepared from liver and kidney and analyzed with cDNA probes specific for Gsst1, Gsst2 and
Gsst3. Gsst4 is expressed at low levels in most tissue. To verify deletion of this gene, we
generated macrophages from bone marrow. We found that Gsst4 was induced after stimulation
with LPS at sufficient levels to allow detection by Northern blot. No expression was observed in
cells derived from GsstΔ/Δ animals (Fig. 3B).

**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
analysis (Supplemental Figure 1C), and these cells were used to generate a GstmΔ/Δ 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 (Fig. 4).

DNA from the homozygous GstmΔ/Δ mice was digested with a number of enzymes and subjected to Southern 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 of 8 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+/Δ mice is consistent with the absence of all of the Gstm genes in these animals. We also verified loss of expression of each of the seven genes. Based both 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 GstmΔ/Δ locus and analyzed by quantitative PCR. 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 GstmΔ/Δ 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 Figure 3). The
deletion includes the entire gene as well as promoter elements, thus mimicking the deletion event that resulted in the null \textit{GSTM1}*0 allele present in humans. We verified that this deletion does not result in loss of expression of the neighboring two genes, particularly \textit{Gstm2} and \textit{Gstm4} (Data not shown).

**Generation of mice lacking \textit{Gstp}, \textit{Gstt} and \textit{Gstm} 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 co-isogenic 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 \textit{Gstt} and \textit{Gstp} loci. This line was then intercrossed with the \textit{GstmΔ/Δ} mice to generate the mice lacking all three gene families. These \textit{GstpΔ/Δ/GstmΔ/Δ/GsttΔ/Δ, (ΔPMT)}, 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 (Supplementary Table 2). Morphological evaluation of tissues showed no gross abnormalities, including in size of organs, nor did histological evaluation reveal an overt impact of loss of these fourteen genes on development (data not shown). For example, histological 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, and 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 (Supplementary 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 129 controls (Supplemental Table 4). Normal blood urea, nitrogen and creatinine indicated normal kidney function, while normal alanine aminotransferase and aspartate aminotransferase levels indicated that loss of these three Gst gene families was not sufficient to induce liver disease in the animals (Supplemental Table 4). The only significant difference observed was a slight decrease in the levels of alkaline phosphatase in the ΔPMT mice. The values, however, were still well within the range observed for mouse (Foreman et al., 2005). Both female and male mice were fertile and raised litters within the normal size range for the 129S6 mouse strain. Taken together, this indicates that these fourteen Gst genes are not required for normal development, maturation and health in a controlled environment, and thus that this line will provide a useful tool for the study of the role of these genes in disease models as well as for the study of their role in metabolism of drugs and xenobiotics.

**Gene expression in ΔPMT mice.** We next asked whether the absence of these three gene families resulted in a compensatory change in the expression of remaining GSTs. Expression of four mouse Gsta1-4 genes, Gstk, Gszt1, and mGst1 was examined in the liver of both male and female ΔPMT animals. Although there was a trend toward increased expression of Gsta genes, particularly in females, none of the changes achieved statistical significance. Also a trend toward increased expression of the microsomal enzyme mGst1 and the peroxisome enzyme Gstk1 was observed in females; however these increases were small and only achieved statistical significance in the case of mGst1. Overall loss of these three gene families had minimal impact on the expression of the remaining GST genes whose expression was examined (Fig. 5).
in the expression of antioxidant enzymes can provide evidence of ongoing oxidative stress. To
determine if such changes were present in the ΔPMT mice, the transcript levels of superoxide
dismutase and glutathione peroxidase were examined (Supplemental Figure 4). This evaluation
indicated that, under normal dietary conditions, loss of the Gstp, Gstm and Gstt gene families did
not result in significant oxidative stress in the liver.

**Metabolism of GST substrates by ΔPMT mice.** GST-CDNB activity is commonly used as an
indicator of total GST activity, and we therefore determined the impact of loss of these fourteen
genes on the activity towards this substrate. Cytoplasmic fractions were prepared from the liver,
lung and kidney of the male and female ΔPMT mice and GST-CDNB activity measured (Fig. 6).
A dramatic decrease in CDNB metabolism was seen in all three tissues in samples prepared from
both male and female ΔPMT mice, with levels of activity in the mutant mice reduced by ~50 to
75% (Fig. 6A). Previous studies using tissue from mouse lines with mutations in Gstp1 and
Gstp2 have demonstrated decreases in the CDNB activity varying from 10% to 40%, depending
on the study, the tissue examined, and the sex of the animals from which the tissue was
harvested. A small decrease in CDNB activity was also reported in mice with a mutant Gstm1
locus. Therefore, we next determined whether the large reduction in the CDNB activity could be
attributed to the combined loss of Gstm1 and the Gstp locus. We observed a small but significant
decrease in the activity of CDNB in the cytosolic extracts from the liver and kidney of the
GstpΔ/Δ mice (Fig. 6C). We however did not observe a difference between these groups in the
CDNB activity in lung extracts as reported for the Gstp1/2-deficient mice. In all cases the
magnitude of the change in activity was small, representing less than 25% of the total CDNB
activity. Similar to previous studies, we observed a decrease in the CDNB activity in extracts
from the Gstm1-/- mice. Therefore, as expected, a decrease was also detected in the GstmΔ/Δ
animals. However, 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 $Gstt$ locus. Consistent with the reported inability of GSTT to metabolize CDNB, no change in CDNB activity was observed in extracts from the $Gstt^{Δ/Δ}$ 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 $APMT$ mice towards DCNB as a substrate preferentially metabolized by GSTM (Fig. 7A). A 90% reduction in activity was observed in extracts from liver, kidney and lung of $APMT$ mice. While no change in activity towards this substrate is observed in extracts from $Gstt^{Δ/Δ}$ mice, a decrease of similar magnitude is observed in the $Gstm^{Δ/Δ}$ mice, assigning the GST-DCNB activity to this family. Evaluation of mice lacking only GSTM1 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 $Gstm1^{-/-}$ animals was not significantly different than the activity of the $Gstm^{Δ/Δ}$ tissues.

The activity towards EPNP is also dramatically reduced in tissues from the $APMT$ mice (Fig. 7B). EPNP is reported to be metabolized primarily by mouse GSTT1. Consistent with this report, comparison of the activity towards this substrate in cytosolic fractions from $Gstt^{Δ/Δ}$ mice and $APMT$ mice assigned all activity to this locus. Similarly, activity toward ethacrynic acid, a substrate for GSTP, was reduced in liver extracts from $APMT$ and $Gstp^{Δ/Δ}$ 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 Gstp, Gstt, and Gstm 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 fourteen genes located in these three major Gst families. In all cases the loss of expression of the Gst 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 carried out to identify conserved, non-coding 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 GsttΔ/Δ 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 which, 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 Gstp1/2 in male mice observed at puberty (Conforto and Waxman, 2012) is not conserved in Gstp3. Second, although we have observed lower transcript levels of BC021614 relative to Gstp1/2 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 show to increase the stability of human GSTP. Final assignment of this gene to the Gstp family awaits determination of whether the protein encoded by BC021614 (Gstp3) has GST activity.

Evaluation of GST activity in the four mouse lines we have generated towards 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 *GstmΔ/Δ* mice and *Gstm1-/-* mice indicates that *Gstm2* through *Gstm7* do not contribute significantly to the metabolism of DCNB, even though the expression of *Gstm2*, *Gstm3*, *Gstm6* and *Gstm4* 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). This substrate may therefore 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 *GstpΔ/Δ* mice demonstrated only a small decrease in activity towards CDNB in liver and kidney, while 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 *Gstp1/2* mice. In an initial study of these animals, Henderson and colleagues reported that the activity towards CDNB was reduced in lung cytosolic fractions but not in the fractions prepared from the kidney and liver (Henderson et al., 1998). However, a later study reported reduced activity towards CDNB in cytosolic extracts prepared form liver, lung and kidney of this 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 co-isogenic 129S6 animals while the initial studies of the *Gstp1/2-/-* mice were carried out with F2 mice carrying 129 and MF1 alleles, while 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
Differences might reflect different environmental conditions including biological differences in the micro flora colonizing skin, intestinal and/or nasal passages, as well as physical difference such as bedding and diet.

Perhaps the most surprising finding of our study of GST activity present in the cytosolic fractions prepared from $\Delta PMT$ mice was the robust and easily repeatable decrease in the metabolism of CDNB by cytosolic fractions prepared from all three tissues, particularly the lung. This change could not be assigned to the $Gstt$ genes, as the $Gstt\Delta/\Delta$ mice showed no change in activity toward CDNB, consistent with other studies. Furthermore, while some of the activity towards CDNB could be assigned to the $Gstm1$ gene, the contribution from this gene represented a small fraction of the total activity. As discussed above, only a small change in activity toward CDNB was observed in the $Gstp\Delta/\Delta$ 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 towards CDNB in the $\Delta PMT$ animals. Taken together, the decreases in activity observed in the $Gstp\Delta/\Delta$ and $Gstm\Delta/\Delta$ mice cannot account for the dramatic decrease in the $\Delta PMT$ animals. One possible explanation is that there is compensatory upregulation of genes in the $Gstp\Delta/\Delta$ and $Gstm\Delta/\Delta$ mice. Compensation for loss of a $Gst$ gene was reported for $Gsta4^{-/-}$ mice, where increased expression of $Gsta2$, $Gsta3$ and $Gstm1$ was observed (Engle et al., 2004). In comparison, we observed no change in $Gsta$, $Gstz1$, or $Gstk1$ expression and only a small increase in $mGst1$ in female $\Delta PMT$ mice. However, we did not examine tissue from the $Gstp\Delta/\Delta$ mice or the $Gstm\Delta/\Delta$ 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 fourteen \textit{Gst} genes were 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 \textit{Gstm}, \textit{Gstp} and \textit{Gstt} genes function primarily in response to changes in environmental stress factors that are largely limited or controlled 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 pharmacological and toxicological studies of the role of these phase II enzymes in the metabolism of drugs and xenobiotics.
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Authorship Contributions

Participation in research design: Snouwaert, Nguyen, Koller

Conducted experiments: Xiang, Snouwaert, Nguyen, Repenning, Latour, Kovarova, Cyphert, Koller

Wrote or contributed to writing of manuscript: Snouwaert, Nguyen, Kovarova, Koller.
References:


Gstm1-null mice by cytosolic and in vivo metabolic studies using 1,2-dichloro-4-nitrobenzene. *Drug Metab Dispos* **34**:1495-1501.


Footnotes:
Zhidan Xiang and John N. Snouwaert contributed equally to this study.

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Figure legends:

**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 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 analysis are shown for each locus. **A.** The mouse *Gstp* locus extends over ~40 kb and is located between the *Cabp2* and *Ndufv1* (not shown) genes on chr19. It includes the two well characterized and highly homologous (91.4 % protein homology) genes, *Gstp1* and *Gstp2*. Located telomeric to *Gstp2* we identified an additional GST-like gene with the current designation *BC021614*, which we here refer to as *Gstp3* based on sequence analysis. The deletion at this locus encompasses chr19:4,028,404-4,067,695 and includes not only the coding regions of the three genes but also approximately 8.5 kb of DNA 5’ of the mouse *Gstp3* and about 7 kb of DNA 3’ of the *Gstp1*. **B.** The mouse *Gstt* locus extends over ~65 kb on chr 10 between *Ddt* and *Mif* (not shown) and includes *Gstt1*, *Gstt2*, *Gstt3*, and *Gstt4*. Because of the close proximity of *Ddt* to *Gstt3* and the possibility that they share regulatory elements, the gene was included in the *Gstt* deletion, which extends from chr10:75,234,558-75,299,968. **C.** Seven *Gstm* genes are located in a ~150 kb segment extending from the *Eps8l3* gene to the *Ampd2* on mouse chr 3. Removal of the entire family of genes and relevant 5’ and 3’ regions required deletion of chr3:107,697,426-107,849,488.
Fig. 2. Comparison of human GSTP1 with mouse GSTP1 and GSTP2 with BC020216
(mGSTP3). A. The primary structure of human GSTP1 is compared to mouse GSTP1 and
GSTP2 and the deduced primary structure of the protein encoded by BC020216 (GSTP3).
Conserved residues that have been implicated in GSTP function are marked by green rectangles.
Tyr 50, a residue at the dimer interface implicated in the cooperativity of the enzyme, is marked
with a red rectangle. This residue is replaced by Phe in GSTP3, a substitution that has been
shown in human GSTP1 to result in higher thermal stability and catalytic efficiency. Numbering
includes the AUG encoded methionine as recommended by the human genome variation society
(www.hgvs.org).

B. Comparison of expression of BC020216 (mGstp3) and Gstp1/2 in liver of male and female
mice. mRNA was isolated from liver of 4 weeks (wk) old male and female mice, sexually mature
male mice (10 wk) and males that were no longer fertile (100 wk) and expression of Gstp1/2 and
Gstp3 compared. The qPCR probes do not distinguish between transcripts originating from
Gstp1 and Gstp2. Expression was normalized to that of 18s RNA. Expression of Gstp1/2 is
significantly higher in the 10 wk old males compared to all other groups. In comparison, no
significant increase is seen in Gstp3 expression between males of different ages (n=3 for all
groups. **p<0.05 determined by unpaired t test.

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 mice GstpΔ/Δ mice and
their controls and analyzed by northern blot using the a full length cDNA probe. The Gstp1
cDNA does not discriminated 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 lanes corresponding to wild type mice but not in the lanes loaded
with RNA from \textit{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 \textit{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 \textit{Gstp1} and \textit{Gstp2} and thus will not hybridize to these transcripts in the conditions used in this study.

Consistent with a deletion that encompasses this telomeric \textit{Gstp} gene, this band is absent in lanes corresponding to the \textit{GstpΔ/Δ} mice. To verify the quality and quantity of the bound RNA filters were probed with \textit{mPges2}, a gene that is expressed at moderate levels throughout the intestinal tract. \textbf{B.} RNA was isolated from liver, kidney, and bone marrow derived macrophages of wild type and \textit{GsttΔ/Δ} mouse. Northern analysis using cDNA probes specific for \textit{Gstt1} and \textit{Gstt2} under stringent hybridization conditions showed high expression of both genes in these tissues. \textit{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 \textit{Gstt} probes in RNA prepared from \textit{GsttΔ/Δ} mice. \textit{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 h 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 \textit{GsttΔ/Δ} mice. \textbf{C.} Total RNA was prepared from the indicated tissues of \textit{GstmΔ/Δ} mice and cohoused sex and age matched controls. Expression of the \textit{Gstm} gene indicated in each of the panels from tissues known to expression each family member was determined by quantitative PCR using gene specific primers obtained from Applied Biosciences. Expression levels were normalized to 18S RNA. Data was analyzed using the comparative C\textsubscript{T} method (\(ΔC_{T}\)) 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 ± SEM.

**Fig. 4. Verification of deletion of the seven mouse Gstm genes by Southern blot analysis.** DNA was prepared from tail biopsies obtained from pups generated by the intercross of GstmΔ/+ mice. DNA was digested with BglII and analyzed by Southern blot using a probe that binds to multiple fragments of carrying Gstm genes located on chr 1. These bands were not observed in DNA obtained from pups Gstm Δ/Δ. The few bands remaining correspond to cross reacting loci present on other chromosomes. For example, the 5.5 kb fragments observed in all lanes corresponds to a segment of DNA on chr 10. See Supplemental Table 1 for assigning all fragments to chromosome and locus.

**Fig. 5. Relative abundance of GST transcripts in the liver of APMT mice.** Expression of the gene indicated in each panel was determined by qPCR of RNA prepared from liver of male and female APMT mice (solid bars) and co-isogenic 129S6 controls (open bars). Expression levels were normalized to 18S RNA. Data was analyzed using the comparative C_T method as described by Applied Biosystems. In all cases the expression is shown as fold change relative to levels in male 129S6 livers. Values represent mean from three animals. Asterisks denote P values: p< 0.05 * determined by unpaired t test.

**Fig. 6. Comparison of GST-CDNB activity in tissues from APMT mice and mice lacking individual GST gene families.** Cytoplasmic fractions were prepared from liver, kidney, and lung of wild type (open bars), APMT, GstpΔ/Δ, GsttΔ/Δ, GstmΔ/Δ and Gstm1-/- mice (solid bars).
and activity towards CDNB determined spectrophotometrically. A. The activity towards CDNB is reduced by 41%, 67% and 75% in the liver, kidney and lung, respectively in the ΔPMT male mice. In female animals the reductions were of similar magnitude (52% in liver, 75% in kidney and 71% in lung). B. A small but significant reduction in activity towards CDNB was observed in the cytoplasmic preparations from kidney and lung the Gstm1−/− and GstmΔ/Δ mice. No difference in activity was observed in this activity when similar subcellular fractions were prepared from the liver of these animals. C. A significant decrease in activity towards CDNB is also observed in cytosol prepared from GstpΔ/Δ liver and kidney (15% and 12% respectively). Values are mean of four animals ± S.E.M. Asterisks indicate P values: p<0.01**; p<0.001*** determined by unpaired t test. All mice were 129S6 co-isogenic between the age of 8 and 16 weeks.

Fig.7. Enzymatic activity of liver, kidney and lung cytosol of ΔPMT mice towards DCNB and EPNP. A. Activity of cytosolic preparations from the three tissues was determined spectrophotometrically. The genotype represented by each bar is indicated in the panel. B. Activity of hepatic cytosol towards EPNP was determined spectrophotometrically. The genotype is shown below each bar. Values represent the mean of four animals ± SEM. Asterisks indicate P values: p<0.05*, p<0.01**; p<0.001*** determined by unpaired t test.
Figure 1

A. **Gstp locus**

- HindIII
- 11.5 kb
- HindIII
- 40 kb deletion
- probe

B. **Gstt locus**

- Cabin1
- Ddt
- Gstt3
- Gstt1
- Gstt4
- Gstt2
- EcoRI
- 8.7 kb
- EcoRI
- 65 kb deletion

C. **Gstm locus**

- Eps8l3
- M5
- M7
- M9
- M1
- M2
- M1
- M4
- Ampd2
- Apal
- 15.8 kb
- Apal
- 150 kb deletion

Probe locations indicated in green.
Figure 2

A

<table>
<thead>
<tr>
<th>hGSTP1</th>
<th>mGSTP1</th>
<th>mGSTP2</th>
<th>mGSTP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPYTVYTFPVRGCAALRM LLADQGQSWEEVTVTDEGSLNASVTLQGLPKFQDDTLTYQSNTILRHRGRTLDL</td>
<td>MPYTVYTFPVRGCAALRM LLADQGQSWEEVTVTDEGSLNASVTLQGLPKFQDDTLTYQSNTILRHRGRTLDL</td>
<td>MPYTVYTFPVRGCAALRM LLADQGQSWEEVTVTDEGSLNASVTLQGLPKFQDDTLTYQSNTILRHRGRTLDL</td>
<td>MPYTVYTFPVRGCAALRM LLADQGQSWEEVTVTDEGSLNASVTLQGLPKFQDDTLTYQSNTILRHRGRTLDL</td>
</tr>
</tbody>
</table>

B

![Graph](image)

Relative Expression

<table>
<thead>
<tr>
<th>Gstp1/2</th>
<th>Gstp3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male 4 w</td>
<td>0.15</td>
</tr>
<tr>
<td>Female 4 w</td>
<td>0.10</td>
</tr>
<tr>
<td>Male 10 w</td>
<td>0.10</td>
</tr>
<tr>
<td>Female 10 w</td>
<td>0.05</td>
</tr>
<tr>
<td>Male 10 w &lt;100 w</td>
<td>0.05</td>
</tr>
</tbody>
</table>

** Significant difference between male and female samples.
Figure 3

A

Gstp1/2 cDNA probe

Gstp3 cDNA probe

mPges2 cDNA probe

+/+

duo colon

duo colon

GstpΔ/Δ

duo colon

duo colon

B

Gstt1 cDNA probe

Gstt2 cDNA probe

mPges2 cDNA probe

+/+
liver kidney

GsttΔ/Δ

liver kidney

Gstt3 cDNA probe

Gstt4 cDNA probe

GsttΔ/Δ

liver kidney

mPges2 cDNA probe

actin cDNA probe

+/+
liver kidney

GsttΔ/Δ

liver kidney

0 LPS

Gstt+/+

0 LPS

GsttΔ/Δ

C

Gstm 1

Gstm 2

Gstm 3

Gstm 4

Gstm 5

Gstm 6

Gstm 7

mPges2

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S

Relative Exp. to 18S
Figure 4

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Figure 5:

Graphs showing relative expression levels of various genes (Gsta1, Gsta2, Gsta3, Gsta4, Gstk1, Gstz1, mGst1) for male and female subjects. The graphs display bar charts with error bars indicating variability.
Figure 6:

A

\( \Delta PMT \) female

\( \Delta PMT \) male

GST-CDNB (nmol/min/mL)

Liver  |  Kidney  |  Lung

Liver  |  Kidney  |  Lung

B

\( Gstm1/-\) male

\( Gstm\Delta/\Delta \) male

GST-CDNB (nmol/min/mL)

Liver  |  Kidney  |  Lung

Liver  |  Kidney  |  Lung

C

\( Gstp\Delta/\Delta \) male

GST-CDNB (nmol/min/mL)

Liver  |  Kidney  |  Lung
Figure 7:

A

GST-DCNB (nmol/min/mL)

Liver  Kidney  Lung

0  100  200  300  400

129  ΔGSTT  GSTM1-/-  ΔGSTM  ΔPMT

***  **  129 ∆GSTT

B

GST-EPNP (nmol/min/mL)

Liver  Kidney  Lung

0  100  200  300  400  500

*  *  129 ∆GSTT

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