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Toxicogenomics of Endoplasmic Reticulum stress inducer Tunicamycin in the Small Intestine and Liver of Nrf2 Knockout and C57BL/6J Mice

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Abstract

This objective of this study was to investigate the toxicogenomics and the spatial regulation of global gene expression profiles elicited by Endoplasmic Reticulum (ER) stress inducer Tunicamycin (TM) in mouse small intestine and liver as well as to identify TM-modulated Nuclear Factor-E2-related factor 2 (Nrf2)–dependent genes. Gene expression profiles were analyzed using 45,000 Affymetrix mouse genome 430 2.0 array and GeneSpring 7.2 software. Microarray results were validated by quantitative real-time reverse transcription-PCR analyses. Clusters of genes that were either induced or suppressed more than two fold by TM treatment compared with vehicle in C57BL/6J/Nrf2(−/−; knockout)and C57BL/6J Nrf2 (+/+; wildtype) mice genotypes were identified. Amongst these, in small intestine and liver, 1291 and 750 genes respectively were identified as Nrf2-dependent and upregulated, and 1370 and 943 genes respectively as Nrf2-dependent and downregulated. Based on their biological functions, these genes can be categorized into molecular chaperones and heat shock proteins, ubiquitination/proteolysis, apoptosis/cell cycle, electron transport, detoxification, cell growth/differentiation, signaling molecules/interacting partners, kinases and phosphatases, transport, biosynthesis/metabolism, nuclear assembly and processing, and genes related to calcium and glucose homeostasis. Phase II detoxification/antioxidant genes as well as putative interacting partners of Nrf2 such as nuclear corepressors and coactivators, were also identified as Nrf2-dependent genes. The identification of TM-regulated and Nrf2-dependent genes in the unfolded protein response to ER stress not only provides potential novel insights into the gestalt biological effects of TM on the toxicogenomics and spatial regulation of global gene expression profiles in cancer pharmacology and toxicology, but also points to the pivotal role of Nrf2 in these biological processes.

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Keywords

Tunicamycin; endoplasmic reticulum stress; Nuclear Factor-E2-related factor 2; microarray; global gene expression profiles

1. Introduction

The endoplasmic reticulum (ER) is an important organelle in which newly synthesized secretory and membrane-associated proteins destined to the extracellular space, plasma membrane, and the exo/endocytic compartments are correctly folded and assembled [1, 2]. An imbalance between the cellular demand for protein synthesis and the capacity of the ER in promoting protein maturation and transport can lead to an accumulation of unfolded or malfolded proteins in the ER lumen. This condition has been designated “ER stress” [2, 3]. Interestingly, the accumulation of misfolded protein in the ER triggers an adaptive stress response – termed the unfolded protein response (UPR) – mediated by the ER transmembrane protein kinase and endoribonuclease inositol-requiring enzyme-1α (IRE1α) [4]. The glucosamine-containing nucleoside antibiotic, Tunicamycin (TM, Fig.1), produced by genus Streptomyces, is an inhibitor of N-linked glycosylation and the formation of N-glycosidic protein-carbohydrate linkages [5]. It specifically inhibits dolichol pyrophosphate-mediated glycosylation of asparaginyl residues of glycoproteins [6] and induces “ER stress”.

Pivotal to the antioxidant response [7–10] typical in mammalian homeostasis and oxidative stress is the important transcription factor Nrf2 or Nuclear Factor-E2-related factor 2 that has been extensively studied by many research groups cited above as well as this laboratory [11–14]. Under homeostatic conditions, Nrf2 is mainly sequestered in the cytoplasm by a cytoskeleton-binding protein called Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Keap1) [11, 15, 16]. When challenged with oxidative stress, Nrf2 is quickly released from Keap1 retention and translocates to the nucleus [11, 17]. We have recently identified [11] a canonical redox-insensitive nuclear export signal (NES) (^{537}LKKQLSTLYL^{546}) located in the leucine zipper (ZIP) domain of the Nrf2 protein as well as a redox-sensitive NES (^{173}LLSI-PELQCLNI^{186}) in the transactivation (TA) domain of Nrf2 [18]. Once in the nucleus, Nrf2 not only binds to the specific consensus cis-element called antioxidant response element (ARE) present in the promoter region of many cytoprotective genes [12, 16, 19], but also to other trans-acting factors such as small Maf (MafG and MafK) [20] that can coordinately regulate gene transcription with Nrf2. We have previously reported [12] that different segments of Nrf2 transactivation domain have different transactivation potential; and that different MAPKs have differential effects on Nrf2 transcriptional activity, with ERK and JNK pathways playing an unequivocal role in positive regulation of Nrf2 transactivation domain activity. To better understand the biological basis of signaling through Nrf2, it has also become imperative to identify possible interacting partners of Nrf2 such as coactivators or corepressors apart from trans-acting factors such as small Maf.

Recently, it was reported [21] that Nrf1, another member of the Cap’ n’ Collar (CNC) family of basic leucine zipper proteins that is structurally similar to Nrf2, is normally targeted to the ER membrane, and that ER stress induced by TM *in vitro* may play a role in modulating Nrf1 function as a transcriptional activator. We sought to investigate the potential role of ER stress in modulating Nrf2 function as a transcriptional activator *in vivo*. Nrf2 knockout mice are greatly predisposed to chemical-induced DNA damage and exhibit higher susceptibility towards cancer development in several models of chemical carcinogenesis [19]. In the present study, we have investigated, by microarray expression profiling, the global gene expression profiles elicited by oral administration of TM in small intestine and liver of Nrf2 knockout (C57BL/6J/Nrf2−/−) and wild type (C57BL/6J) mice to enhance our understanding of TM-

regulated toxicological effects mediated through Nrf2. We have identified clusters of TM-modulated genes that are Nrf2-dependent in small intestine and liver and categorized them based on their biological functions. The identification of TM-regulated Nrf2-dependent genes will yield valuable insights into the role of Nrf2 in TM-modulated gene regulation with respect to cancer pharmacology and toxicology. This study also enables the identification of novel molecular targets that are regulated by TM *via* Nrf2. The current study is also the first to investigate the global gene expression profiles elicited by TM in an *in vivo* murine model where the role of Nrf2 is also examined.

2.0. Materials and Methods

2.1. Animals and Dosing

The protocol for animal studies was approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC). Nrf2 knockout mice Nrf2 (−/−) (C57BL/SV129) have been described previously.[22]. Nrf2 (−/−) mice were backcrossed with C57BL/6J mice (The Jackson Laboratory, ME USA). DNA was extracted from the tail of each mouse and genotype of the mouse was confirmed by polymerase chain reaction (PCR) by using primers (3′-primer, 5′-GGA ATG GAA AAT AGC TCC TGC C-3′; 5′-primer, 5′-GCC TGA GAG CTG TAG GCC C-3′; and lacZ primer, 5′-GGG TTT TCC CAG TCA CGA C-3′). Nrf2(−/−) mice-derived PCR products showed only one band of ~200bp, Nrf2 (+/+) mice-derived PCR products showed a band of ~300bp while both bands appeared in Nrf2(+/−) mice PCR products. Female C57BL/6J/Nrf2(−/−) mice from third generation of backcrossing were used in this study. Age-matched female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice in the age-group of 9–12 weeks were housed at Rutgers Animal Facility with free access to water and food under 12 h light/dark cycles. After one week of acclimatization, the mice were put on AIN-76A diet (Research Diets Inc. NJ USA) for another week. The mice were then administered TM (Sigma-Aldrich, St.Louis, MO) at a dose of 2 mg/kg (dissolved in 50% PEG 400 aqueous solution) by oral gavage. The control group animals were administered only vehicle (50% PEG 400 aqueous solution). Each treatment was administrated to a group of four animals for both C57BL/6J and C57BL/6J/Nrf2(−/−) mice. Mice were sacrificed 3h after TM treatment or 3 h after vehicle treatment (control group). Livers and small intestines were retrieved and stored in RNA Later (Ambion, Austin,TX) solution.

2.2. Sample Preparation for Microarray Analyses

Total RNA from liver and small intestine tissues were isolated by using a method of TRIzol (Invitrogen, Carlsbad, CA) extraction coupled with the RNeasy kit from Qiagen (Valencia, CA). Briefly, tissues were homogenized in trizol and then extracted with chloroform by vortexing. A small volume (1.2 ml) of aqueous phase after chloroform extraction and centrifugation was adjusted to 35% ethanol and loaded onto an RNeasy column. The column was washed, and RNA was eluted following the manufacturer's recommendations. RNA integrity was examined by electrophoresis, and concentrations were determined by UV spectrophotometry.

2.3. Microarray Hybridization and Data Analysis

Affymetrix (Affymetrix, Santa Clara, CA) mouse genome 430 2.0 array was used to probe the global gene expression profiles in mice following TM treatment. The mouse genome 430 2.0 Array is a high-density oligonucleotide array comprised of over 45,101 probe sets representing over 34,000 well-substantiated mouse genes. The library file for the above-mentioned oligonucleotide array is readily available at http://www.affymetrix.com/support/technical/libraryfilesmain.affx. After RNA isolation, all the subsequent technical procedures including quality control and concentration measurement of RNA, cDNA synthesis and biotin-labeling of cRNA, hybridization and scanning of the

arrays, were performed at CINJ Core Expression Array Facility of Robert Wood Johnson Medical School (New Brunswick, NJ). Each chip was hybridized with cRNA derived from a pooled total RNA sample from four mice per treatment group, per organ, and per genotype (a total of eight chips were used in this study) (Fig.2). Briefly, double-stranded cDNA was synthesized from 5 μg of total RNA and labeled using the ENZO BioArray RNA transcript labeling kit (Enzo Life Sciences, Inc.,Farmingdale, NY, USA) to generate biotinylated cRNA. Biotin-labeled cRNA was purified and fragmented randomly according to Affymetrix's protocol. Two hundred microliters of sample cocktail containing 15 μg of fragmented and biotin-labeled cRNA was loaded onto each chip. Chips were hybridized at 45°C for 16 h and washed with fluidics protocol EukGE-WS2v5 according to Affymetrix's recommendation. At the completion of the fluidics protocol, the chips were placed into the Affymetrix GeneChip Scanner where the intensity of the fluorescence for each feature was measured. The expression value (average difference) for each gene was determined by calculating the average of differences in intensity (perfect match intensity minus mismatch intensity) between its probe pairs. The expression analysis file created from each sample (chip) was imported into GeneSpring 7.2 (Agilent Technologies, Inc., Palo Alto, CA) for further data characterization. Briefly, a new experiment was generated after importing data from the same organ in which data was normalized by array to the 50th percentile of all measurements on that array. Data filtration based on flags present in at least one of the samples was first performed, and a corresponding gene list based on those flags was generated. Lists of genes that were either induced or suppressed more than two fold between treated versus vehicle group of same genotype were created by filtration-on-fold function within the presented flag list. By use of color-by-Venn-Diagram function, lists of genes that were regulated more than two fold only in C57BL/6J mice in both liver and small intestine were created. Similarly, lists of gene that were regulated over two fold regardless of genotype were also generated.

2.4. Quantitative Real-time PCR for Microarray Data Validation

To validate the microarray data, several genes of interest were selected from various categories for quantitative real-time PCR analyses. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) served as the "housekeeping" gene. The specific primers for these genes listed in Table I were designed by using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and were obtained from Integrated DNA Technologies, Coralville, I A. The specificity of the primers was examined by a National Center for Biotechnology Information Blast search of the mouse genome. Instead of using pooled RNA from each group, RNA samples isolated from individual mice as described earlier were used in real-time PCR analyses. For the real-time PCR assays, briefly, first-strand cDNA was synthesized using 4μg of total RNA following the protocol of SuperScript III First-Strand cDNA Synthesis System (Invitrogen) in a 40 μl reaction volume. The PCR reactions based on SYBR Green chemistry were carried out using 100 times diluted cDNA product, 60 nM of each primer, and SYBR Green master mix (Applied Biosystems, Foster City, CA) in 10 μl reactions. The PCR parameters were set using SDS 2.1 software (Applied Biosystems, Foster City, CA) and involved the following stages : 50°C for 2min, 1 cycle; 95°C for 10 mins, 1 cycle; 95°C for 15 secs → 55 °C for 30 secs → 72°C for 30 secs, 40 cycles; and 72°C for 10 mins, 1 cycle. Incorporation of the SYBR Green dye into the PCR products was monitored in real time with an ABI Prism 7900HT sequence detection system, resulting in the calculation of a threshold cycle (C_T) that defines the PCR cycle at which exponential growth of PCR products begins. The carboxy-X-rhodamine (ROX) passive reference dye was used to account for well and pipetting variability. A control cDNA dilution series was created for each gene to establish a standard curve. After conclusion of the reaction, amplicon specificity was verified by first-derivative melting curve analysis using the ABI software; and the integrity of the PCR reaction product and absence of primer dimers was ascertained. The gene expression was determined by normalization with control gene GAPDH.

In order to validate the results, the correlation between corresponding microarray data and real-time PCR data was evaluated by the statistical 'coefficient of determination', $\mathbf{r^2}$=0.97.

3.0. Results

3.0.1. TM-Modulated Gene Expression Patterns in Mouse Small Intestine and Liver

Subsequent to data normalization, 48.76% (21,991) of the probes passed the filtration based on flags present in at least one of four small intestine sample arrays depicted in Figure 2. Expression levels of 1291 probes were elevated or of 1370 probes were suppressed over two fold by TM only in the wild-type mice, while 3471 probes were induced or 2024 probes were inhibited over two fold by TM only in the Nrf2(−/−) mice small intestine (Fig.3a). Similarly, changes in gene expression profiles were also observed in mice liver. Overall, the expression levels of 51.495% (23,225) probes were detected in least in one of four liver sample arrays depicted in Figure 2. In comparison with the results from small intestine sample arrays, a smaller proportion of well-defined genes were either elevated (750) or suppressed (943) over two fold by TM in wild-type mice liver alone; whereas 39 well-defined genes were induced or 3170 genes were inhibited in Nrf2(−/−) mice liver. (Fig.3b).

3.0.2. Quantitative Real–Time PCR Validation of Microarray Data

To validate the data generated from the microarray studies, several genes from different categories (Table I) were selected to confirm the TM-regulative effects by the use of quantitative real-time PCR analyses as described in detail under Materials and Methods. After ascertaining the amplicon specificity by first-derivative melting curve analysis, the values obtained for each gene were normalized by the values of corresponding GAPDH expression levels. The fold changes in expression levels of treated samples over control samples were computed by assigning unit value to the control (vehicle) samples. Computation of the correlation statistic showed that the data generated from the microarray analyses are well-correlated with the results obtained from quantitative real-time PCR (coefficient of determination, $r^2 = 0.97$, Fig.4).

3.0.3. TM-Induced Nrf2-Dependent Genes in Small Intestine and Liver

Genes that were induced only in wild-type mice, but not in Nrf2(−/−) mice, by TM were designated as TM-induced Nrf2-dependent genes. Based on their biological functions, these genes were classified into categories, including ubiquitination and proteolysis, electron transport, chaperones and unfolded protein response genes, detoxification enzymes, transport, apoptosis and cell cycle control, cell adhesion, kinases and phosphatases, transcription factors and interacting partners, glucose-related genes, ER and Golgi-related genes, translation factors, RNA/Protein processing and nuclear assembly, biosynthesis and metabolism, cell growth and differentiation, and G protein-coupled receptors (Table II lists genes relevant to our interest).

In response to TM-induced ER stress, several unfolded protein response genes were identified as Nrf2-regulated including, amongst others, heat shock protein, alpha-crystallin-related, B6 (Hspb6) in liver, heat shock protein family, member 7,cardiovascular (Hspb7) in small intestine, and stress 70 protein chaperone, microsome-associated, human homolog (Stch) in both liver and small intestine. A large number of apoptosis and cell-cycle related genes were also upregulated in response to TM treatment. Representative members included B-cell leukemia/lymphoma 2 (Bcl2), CASP8 and FADD-like apoptosis regulator (Cflar), Epiregulin (Ereg), Growth arrest specific 2 (Gas2) and synovial apoptosis inhibitor 1, synoviolin (Syvn1). Interestingly, several important transcription/translation factors and interacting partners were identified as Nrf2-dependent and TM-regulated. These included P300/CBP-associated factor (Pcaf), Smad nuclear interacting protein 1 (Snip1), nuclear receptor coactivator 5 (Ncoa5), nuclear receptor interacting protein 1 (Nrip1), nuclear transcription factor, X-box binding-like

1 (Nfxl1), eukaryotic translation initiation factors 1α 2, 4e and 5 (Eif 1a2, 4e and 5), Erbb2 interacting protein (Erbb2ip), cAMP responsive element binding protein 3-like 2 (Creb3l2) and Jun oncogene (Jun).

Other categories of genes induced by TM in an Nrf2-dependent manner included cell adhesion (cadherins 1, 2, and 10), glucose-related genes (hexokinase 2), transport (solute carrier family members Slc13a1, Slc22a3, Slc8a1 and others), and ubiquitination and proteolysis (Constitutive photomorphogenic protein and carboxypeptidase A4). The glutathione peroxidase 3 (Gpx3) gene was also upregulated in liver in an Nrf2-dependent manner in response to TM treatment.

3.0.4. TM-Suppressed Nrf2-Dependent Genes in Small Intestine and Liver

As shown in Table III which lists genes relevant to our interest, TM treatment also inhibited the expression of many genes falling into similar functional categories in an Nrf2-dependent manner. Major Phase II detoxifying genes identified as Nrf2-regulated and TM-modulated included several isoforms of Glutathione-S-transferase (Gst), and glutamate cysteine ligase, modifier subunit (Gclm). Additionally, Phase I genes such as cytochrome P450 family members Cyp3a44, Cyp39a1 and Cyp8b1 were also downregulated in response to TM-treatment in an Nrf2-dependent manner. Moreover, many transport genes, which may be regarded as Phase III genes, including members of solute carrier family (Slc23a2, Slc23a1, Slc37a4, Slc4a4, Slc40a1, Slc9a3) and multidrug-resistance associated proteins (Abcc3) were also downregulated *via* Nrf2 and regulated through TM. Thus, a co-ordinated response involving Phase I, II and III genes was observed on TM treatment in an Nrf2-dependent manner.

Other categories of genes affected included apoptosis and cell cycle-related genes (Caspases 6 and 11, growth arrest and DNA-damage-inducible 45 β), electron transport (Cyp450 members and NADH dehydrogenase isoforms), kinases and phosphatases (mitogen activated protein kinase family members, ribosomal protein S6 kinase), transcription factors and interacting partners (inhibitor of kappa B kinase gamma and src family associated phosphoprotein 2), and glucose-related genes (glucose-6-phosphatase, catalytic, fructose bisphosphatase 1, and glucose phosphate isomerase 1). Superoxide dismutase (Sod1) was also identified as an Nrf2-regulated and TM-modulated gene that was suppressed. Furthermore, cell adhesion genes (cadherin 22), ubiquitination and proteolysis genes (Usp25 and Usp34), and some unfolded protein response genes (heat shock proteins 1B and 3) were also observed to be downregulated in response to TM treatment *via* Nrf2.

4.0. Discussion

The major goal of this study was to identify toxicant Tunicamycin-regulated Nrf2-dependent genes in mice liver and small intestine by using C57BL/6J Nrf2 (+/+; wildtype) and C57BL/6J/Nrf2(−/−; knockout) mice and genome-scale microarray analyses. We sought to investigate by transcriptome expression profiling the potential role of ER stress stimulus in modulating Nrf2 function as a transcriptional activator *in vivo*. As a protein-folding compartment, the ER is exquisitely sensitive to alterations in homeostasis, and provides stringent quality control systems to ensure that only correctly folded proteins transit to the Golgi and unfolded or misfolded proteins are retained and ultimately degraded. A number of biochemical and physiological stimuli, such as perturbation in calcium homeostasis or redox status, elevated secretory protein synthesis, expression of misfolded proteins, sugar/glucose deprivation, altered glycosylation, and overloading of cholesterol can disrupt ER homeostasis, impose stress to the ER, and subsequently lead to accumulation of unfolded or misfolded proteins in the ER lumen [23]. The ER has evolved highly specific signaling pathways called the unfolded protein response (UPR) to cope with the accumulation of unfolded or misfolded proteins [4, 23]. ER stress stimulus by Thapsigargin has also been shown [24] to activate the c-Jun N-terminal kinase

(JNK) or stress-activated protein kinase (SAPK) that is a member of the mitogen-activated protein kinase (MAPK) cascade [25]. Moreover, it has been reported that the coupling of ER stress to JNK activation involves transmembrane protein kinase IRE1 by binding to an adaptor protein TRAF2, and that IRE1$\alpha^{-/-}$ fibroblasts were impaired in JNK activation by ER stress [26]. We have previously reported that phenethyl isothiocyanate (PEITC) from cruciferous vegetables activates JNK1 [27] and that the activation of the antioxidant response element (ARE) by PEITC involves both Nrf2 and JNK1 [13] in HeLa cells. We have also reported [12] that extracellular signal-regulated kinase (ERK) and JNK pathways play an unequivocal role in positive regulation of Nrf2 transactivation domain activity *in vitro* in HepG2 cells. Recently, it was shown [21] that Nrf1, another member of the Cap' n' Collar (CNC) family of basic leucine zipper proteins that is structurally similar to Nrf2, is normally targeted to the ER membrane, and that ER stress induced by TM *in vitro* may play a role in modulating Nrf1 function as a transcriptional activator. Here, we investigated the role of Nrf2 in modulating transcriptional response to ER stress stimulus by TM *in vivo* in an Nrf2 (−/−; deficient) murine model, thus providing new biological insights into the diverse cellular and physiological processes that may be regulated by the UPR in cancer pharmacology and toxicology.

Interestingly, a co-ordinated response involving Phase I, II and III genes that has not been demonstrated earlier was observed *in vivo* on ER stress induction with TM in an Nrf2-dependent manner. Phase I drug-metabolizing enzymes (DMEs) such as cytochrome P450 family members Cyp3a44, Cyp39a1 and Cyp8b1 were downregulated in response to TM-treatment in an Nrf2-dependent manner. Additionally, major Phase II detoxifying genes identified as Nrf2-regulated and TM-modulated included several isoforms of Glutathione-S-transferase (Gst), and glutamate cysteine ligase, modifier subunit (Gclm). Moreover, many transport genes, which may be regarded as Phase III genes, including members of solute carrier family (Slc23a2, Slc23a1, Slc37a4, Slc4a4, Slc40a1, Slc9a3) and multidrug-resistance associated proteins (Abcc1, Abcc3 and Mdr1b or Abcb1b) were also downregulated *via* Nrf2 and regulated through TM. The co-ordinated regulation of these genes could have significant effects in toxicology by enhancing the cellular defense system, preventing the activation of procarcinogens/reactive intermediates, and increasing the excretion/efflux of reactive carcinogens or metabolites.

There could be two possible outcomes of prolonged ER stress: (1) an adaptive response promoting cell survival; or (2) the induction of apoptotic cell death [3]. Indeed, several genes related to apoptosis and cell cycle control were modulated in response to TM stimulus *in vivo* in an Nrf2-dependent manner. The major genes upregulated in this category included the anti-apoptotic B-cell leukemia/lymphoma 2 (Bcl2) family gene, CASP8 and FADD-like apoptosis regulator (Cflar), Epiregulin (Ereg), Growth arrest specific 2 (Gas2), cyclin T2 (Ccnt2) and cyclin-dependent kinase 7 (Cdk7) all in small intestine apart from mucin 20 (Muc20) and synovial apoptosis inhibitor 1, synoviolin (Syvn1) in liver ; whereas genes downregulated in this category included cyclin-dependent kinase 6 (Cdk6) and Bcl2 in liver, baculoviral inhibitor of apoptosis (IAP)-repeat containing 6 (Birc6) and Caspases 6 and 11 in small intestine, and growth arrest and DNA-damage-inducible 45 – β (Gadd45b), and gamma interacting protein 1 (Gadd45gip1) - in liver and small intestine respectively amongst others. To our knowledge, this is the first report *in vivo* of apoptosis and cell cycle-related genes that are both modulated by the ER stress inducer TM and regulated *via* Nrf2. Moreover, it has been noted [28] that although the basic machinery to carry out apoptosis appears to be present in essentially all mammalian cells at all times, the activation of the suicide program is regulated by many different signals that originate from both the intracellular and the extracellular milieu. Notably, transcription factor NF-κB is critical for determining cellular sensitivity to apoptotic stimuli by regulating both mitochondrial and death receptor apoptotic pathways. Recently, it was reported [29] that autocrine tumor necrosis factor alpha links ER stress to the membrane

death receptor pathway through IRE1alpha-mediated NF-κB activation and down-regulation of TRAF2 expression. In our study, we saw a downregulation of inhibitor of kappaB kinase gamma (Iκbkg or IKKγ) in liver in an Nrf2-dependent manner in response to TM-induced ER stress. Since the catalytic subunits, IKK and IKKβ, require association with the regulatory IKKγ (NEMO) component to gain full basal and inducible kinase activity and since tetrameric oligomerization of IκB Kinase γ (IKKγ) is obligatory for IKK Complex activity and NF-κB activation [30], our results appear to be validated from a functional standpoint and underscore the complexity of factors involved in making the decision between cell survival and cell death in response to TM-mediated ER stress *in vivo*, not excluding the possibility of potential cross-talk between Nrf2/ARE pathway and other signaling pathways that may converge at multiple levels in the cell.

Interestingly, impaired proteasome function through pharmacological inhibition, or by accumulation of malfolded protein in the cytoplasm, can ultimately block ER-associated degradation (ERAD) [31] which is important for eviction of malfolded proteins from the ER to the cytoplasm where they are subsequently ubiquitinated and degraded *via* the proteasome. In our study, several genes associated with the ubiquitin/proteasome pathway were regulated in response to TM in an Nrf2-dependent manner. These included, amongst others, constitutive photomorphogenic protein (Cop1), carboxypeptidase A4 (Cpa4), ubiquitin-specific peptidase 34 (Usp34), and ubiquitin-specific processing protease (Usp25). Furthermore, UPR genes such as various heat shock proteins (Hspb3, Hspb6, Hspb7, Hspa1B) and molecular chaperones and folding enzymes, e.g., stress 70 protein chaperone (Stch) were also seen to be regulated by TM-induced ER stress and modulated by Nrf2. Since the UPR directs gene expression important for remediating accumulation of malfolded protein in the ER, the identification of UPR-responsive genes in our study validates our results from a biological perspective. Moreover, important genes related to glycosylation modifications (e.g., galactosyltransferase, B3galt1), ER to Golgi transport (ADP-ribosylation factor GTPase activating protein 3, Arfgap3; coatomer protein complex subunit alpha, Copa; Lectin, mannose-binding 1,Lman1), and intra-Golgi transport (Golgi associated, gamma adaptin ear containing, ARF binding protein 2, Gga2) were also seen to be regulated by TM in an Nrf2-dependent manner. Genes related to biogenesis of ribosomes on rough ER where proteins are synthesized from mRNA, e.g., brix domain containing 2 (Bxdc2) and ribosomal protein S6 kinase, polypeptides 1 (Rps6ka1) and 4 (Rps6ka4), were also regulated *via* Nrf2 and modulated by TM treatment. To our knowledge, this is the first *in vivo* investigation examining the potential role of Nrf2 and TM-induced ER stress in the simultaneous modulation of UPR-responsive genes, clearance by the ubiquitin/proteasome pathway members, and cellular biosynthetic-secretory pathway involving ribosomal biogenesis genes and ER to Golgi transport genes.

Additionally, many genes related to glucose biosynthesis and metabolism including glucose phosphate isomerase 1 (gluconeogenesis/glycolysis), fructose bisphosphatase 1 (gluconeogenesis), glucose-6-phosphatase (glycogen biosynthesis), hexokinase 2 (glycolysis), adiponectin (glucose metabolism), lectins (galactose- and mannose-binding) and the solute carrier family member Slc 35b1 (sugar porter) were all seen to be regulated through Nrf2 and modulated by TM-induced ER stress. The simultaneous modulation of genes encoding for insulin like growth factor receptors 1 and 2 point to a potential role for glucose- and ER stress-mediated insulin resistance [32] wherein the potential role of Nrf2 has never been examined earlier.

In recent times, there is a renewed interest in dissecting the interacting partners of Nrf2 such as coactivators and corepressors which are co-regulated with Nrf2 to better understand the biochemistry of Nrf2. In a recent microarray study [33], we have reported that CREB-binding protein (CBP) was upregulated in mice liver on treatment with (-)epigallocatechin-3-gallate (EGCG) in an Nrf2-dependent manner. We have also demonstrated [12] previously, using a

Gal4-Luc reporter co-transfection assay system in HepG2 cells, that the nuclear transcriptional coactivator CBP, which can bind to Nrf2 transactivation domain and can be activated by extracellular signal-regulated protein kinase (ERK) cascade, showed synergistic stimulation with Raf on the transactivation activities of both the chimera Gal4-Nrf2 (1–370) and the full-length Nrf2. In the current study, we observed the upregulation of the P300/CBP-associated factor (P/CAF), transacting factor v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (Maf F), nuclear receptor co-activator 5 (Ncoa5), nuclear receptor co-repressor interacting protein (Nrip1) and Smad nuclear interacting protein 1 (Snip1) ; as well as downregulation of the src family associated phosphoprotein 2 (Scap2) in an Nrf2-dependent manner. Although microarray expression profiling cannot provide evidence of binding between partners, this is the first investigation to potentially suggest that co-repressor Nrip1 and co-activators P/CAF and Ncoa5, similar to CBP in our previous studies, may serve as putative TM-regulated nuclear interacting partners of Nrf2 in eliciting the UPR-responsive events *in vivo*. We have also shown recently [34] that coactivator P/CAF could transcriptionally activate a chimeric Gal4-Nrf2-Luciferase system containing the Nrf2 transactivation domain in HepG2 cells. In addition, P/CAF which is known [35] to be a histoneacetyl transferase protein has recently been shown [36] to mediate DNA damage-dependent acetylation on most promoters of genes involved in the DNA-damage and ER-stress response, which validates our observation of P/CAF induction *via* Nrf2 in response to TM-induced ER stress. Taken together, it is tempting to speculate that the TM-regulated pharmacological and toxicological effects may be regulated by a multimolecular complex, which involves Nrf2 along with the transcriptional co-repressor Nrip1 and the transcriptional co-activators P/CAF and Ncoa5, in addition to the currently known trans-acting factors such as small Maf [20], with multiple interactions between the members of the putative complex as we have shown recently with the p160 family of proteins [34]. Indeed, further studies of a biochemical nature would be needed to substantiate this hypothesis and extend our understanding of Nrf2 regulation in TM-mediated ER stress.

Many important transcription factors affecting diverse signaling pathways were identified as regulated through Nrf2 and modulated by TM treatment. For example, Jun oncogene, platelet-derived growth factor, metallothionein 1 and 2, transforming growth factor beta 1 and ErbB2 interacting protein were upregulated ; whereas hypoxia-inducible factor 1, alpha subunit inhibitor, peroxisome proliferator activated receptor binding protein, v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) and protein kinase C binding protein 1 were downregulated *via* Nrf2 in response to TM. Since these transcription factors can modulate the expression of many different gene transcripts encoding various proteins, their identification as Nrf2-regulated and ER-stress- or TM-modulated would be important in enhancing our current understanding of UPR responsive genes and in providing new biological insights into the diverse cellular and physiological processes that may be regulated by the UPR in Nrf2-regulated cancer pharmacology and toxicology.

In the category of kinases and phosphatases, several members of the MAPK cascade such as Map2k7, Mapk14, Mapk8, Map3k7 as well as MAPK-activated protein kinase 5 (Mapkapk5) were identified as regulated by TM *via* Nrf2. Moreover, members of the calcium/calmodulin signaling pathway such as calcium/calmodulin-dependent - protein kinase I gamma (Camkg), -protein kinase 1D (Camk1d) and -protein kinase IV (Camk4) were shown to be regulated by TM in an Nrf2-dependent manner. Interestingly, glutathione peroxidase 3 (Gpx3) was upregulated and superoxide dismutase 1 (Sod1) was downregulated by TM *via* Nrf2 which can have important implications in oxidative stress-mediated [37] pathophysiology or ER stress caused by perturbations in redox circuitry [23, 37, 38]

Indeed, there is a growing interest amongst researchers in targeting the UPR in cancerous tumor growth [39]. Recently, it was shown [40] that the proteasomal inhibitor bortezomib induces a unique type of ER stress characterized by an absence of eif2alpha phosphorylation,

ubiquitylated protein accumulation, and proteotoxicity in human pancreatic cancer cells. It was also reported [41] that malignant B cells may be highly dependent on ER-Golgi protein transport and that targeting and inhibiting this process by brefeldin A may be a promising therapeutic strategy for B-cell malignancies, especially for those that respond poorly to conventional treatments, e.g., fludarabine resistance in chronic lymphocytic leukemia (CLL). However, the role of Nrf2 in modulating the UPR *in vivo* has never been examined before.

The current study, thus, addresses the spatial regulation in mouse small intestine and liver of global gene expression profiles elicited by TM-mediated ER stress *via* Nrf2. Several common clusters of genes such as that for ubiquitin/proteasome, cell adhesion, transcription factors were observed in this study that were also observed in previous studies with Nrf2 activators[9, 33, 42–44] which validates our studies from a functional standpoint. In addition, three clusters of genes – calcium homeostasis, ER/Golgi transport & ER/Golgi biosynthesis/metabolism genes, and glucose homeostasis genes – were uniquely observed as modulated via Nrf2 in response to TM-mediated ER stress that were not discernible in previous studies with Nrf2 activators. Indeed, the involvement of the three clusters mentioned above is a rational response to alteration in the homeostatic environment brought about by the toxicant TM-induced ER stress, and is reflective of their potential role in the UPR to ER stress that is naturally not observed in previous studies on cancer chemoprevention with Nrf2 activators that do not induce ER stress. The presence of the three unique clusters as mentioned above that relate to the putative role of these genes in the UPR is an effect that appears to be elicited in a toxicant-specific manner. In addition, classical Phase II genes such as Gst isoforms and Gclm were downregulated in a Nrf2-dependent fashion in response to the toxicant TM at 3 hours in this study. We were able to see the downregulation of classical Phase II genes in qRT-PCR experiments performed at a 12 hour time-point (data not shown) with the extent of downregulation being more pronounced at 12 hours than at 3 hours in response to the toxicant TM. Interestingly, this contrasts with the delayed response reported for the classical Phase II gene NQO1 in response to Nrf2 activator BHA (Butylated hydroxyanisole) wherein the induction of the gene peaked at 12 hours[43] with no gene induction at 3 hours. Taken together, the downregulation of classical Phase II genes in response to TM-induced ER stress should be viewed in the light of a complex of physiological factors including partitioning across the gastrointestinal tract, intestinal transit time, uptake into the hepatobiliary circulation, exposure parameters such as Cmax, Tmax and AUC, and pharmacokinetics of disposition after oral administration of TM. Further studies will be necessary to address the effect(s) of temporal dependence on pharmacokinetic parameters and gene expression profiles to further enhance our current understanding of TM-mediated ER stress response, the complexity of kinetics of Phase II gene expression response to a toxicant and the role of Nrf2.

In conclusion, our microarray expression profiling study provides some novel insights into the pharmacogenomics and spatial regulation of global gene expression profiles elicited in the mouse small intestine and liver by TM in an Nrf2-dependent manner from a biological perspective. Amongst these TM-regulated genes, clusters of Nrf2-dependent genes were identified by comparing gene expression profiles between C57BL/6J Nrf2(+/+) and C57BL/6J/Nrf2(−/−) mice. The identification of novel molecular targets that are regulated by TM *via* Nrf2 *in vivo* raises possibilities for targeting the UPR proteins in future to augment or suppress the ER stress response and modulate disease progression. This study clearly extends the current latitude of thought on the molecular mechanisms underlying TM-mediated UPR effects as well as the role(s) of Nrf2 in its biological functions. Future *in vivo* and *in vitro* mechanistic studies exploring the germane molecular targets or signaling pathways as well as Nrf2-dependent genes related to the significant functional categories uncovered in the current study would greatly extend our understanding of the diverse cellular and physiological processes that may be regulated by the UPR in cancer pharmacology and toxicology, and the potential role of ER stress in modulating Nrf2 function as a transcriptional activator. .

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ABBREVIATIONS

TM
: Tunicamycin

ER
: Nuclear Factor-E2 -related factor 2, Nrf2, Endoplasmic Reticulum

UPR
: Unfolded Protein Response

Mapk
: Mitogen-activated protein kinase

ARE
: Antioxidant response element

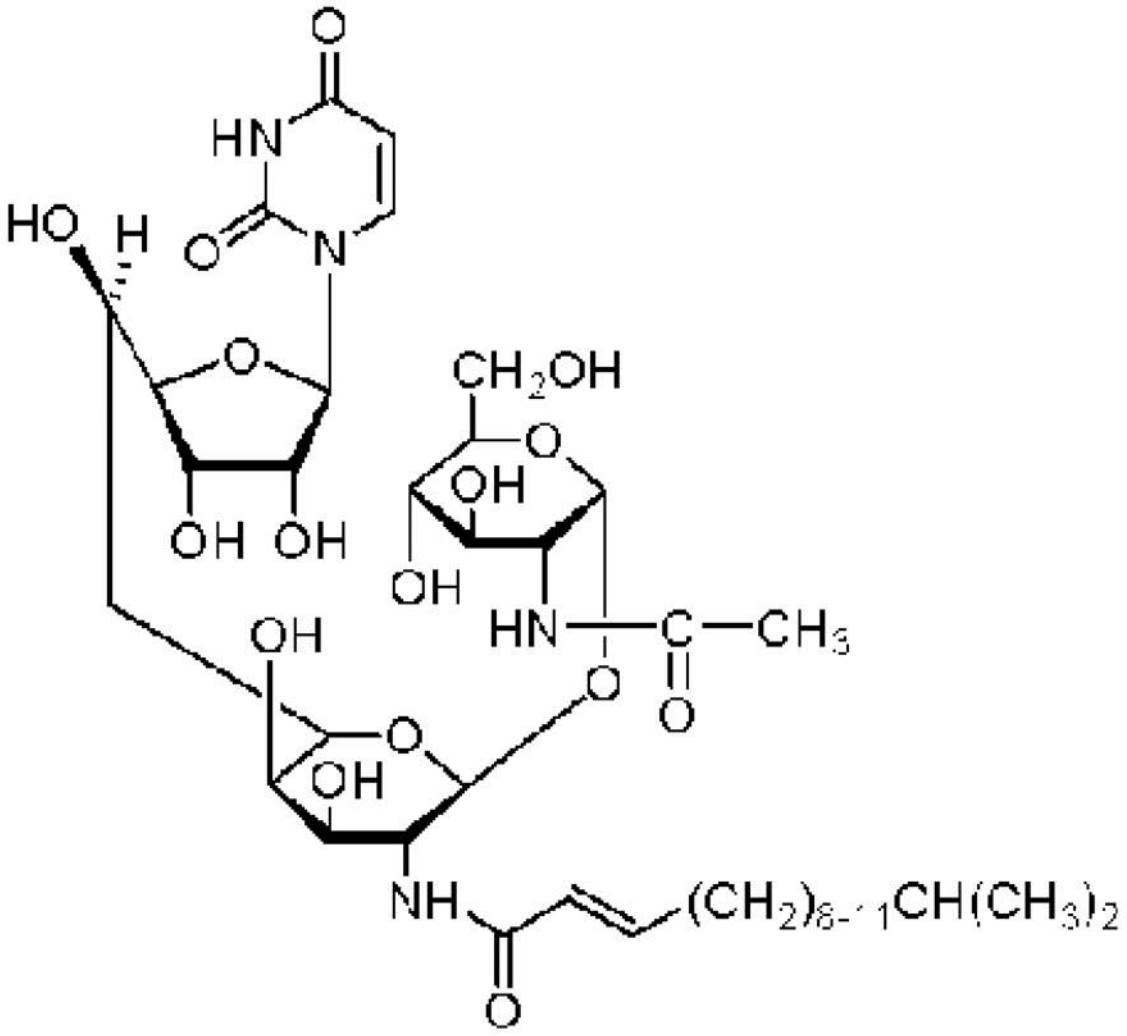

Fig. 1.
Chemical Structure of Tunicamycin (TM).

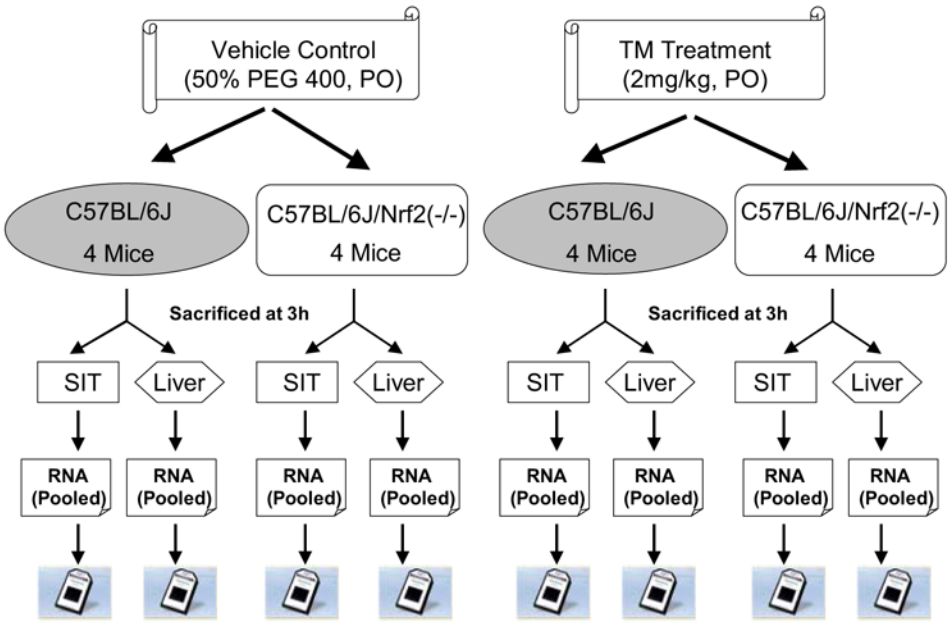

Fig. 2.
Schematic representation of experimental design; *SIT*, Small Intestine.

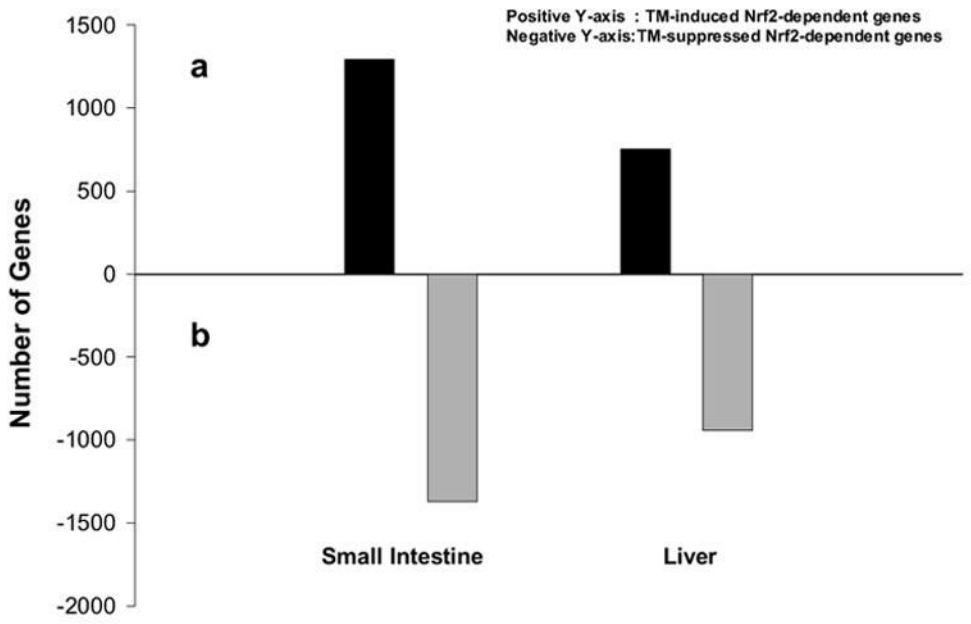

Fig. 3. Regulation of Nrf2-dependent gene expression by TM in mouse small intestine and liver

Gene expression patterns were analyzed at 3h after administration of a 2mg/kg single oral dose of TM; Nrf2-dependent genes that were either induced or suppressed over two fold were listed. The positive numbers on the *y-axis* refer to the number of genes being induced; the negative numbers on the *y-axis* refer to the number of genes being suppressed.

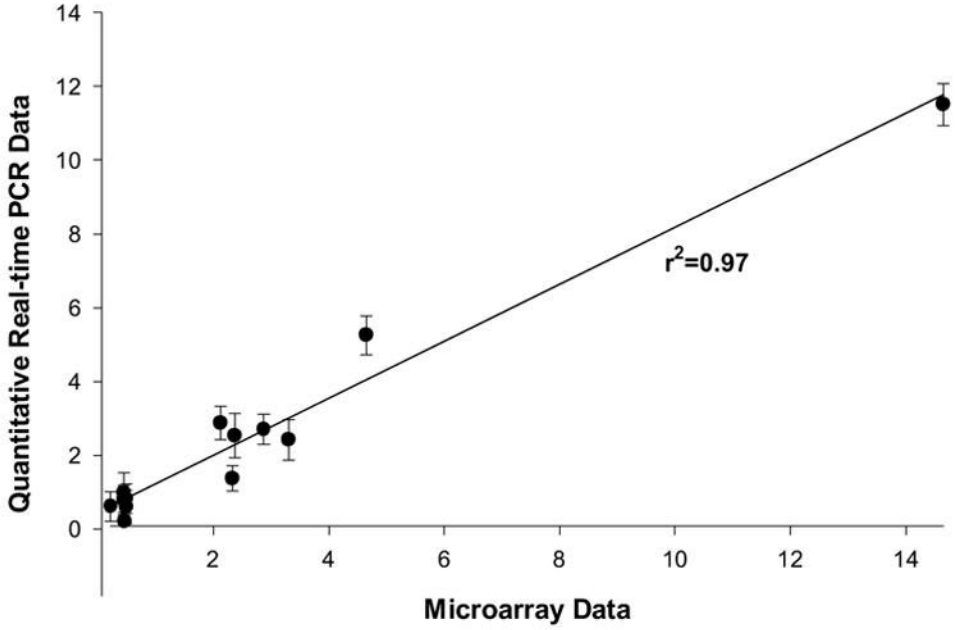

Fig. 4. Correlation of microarray data with quantitative real-time PCR data

Fold changes in gene expression measured by quantitative real-time PCR for each sample in triplicate (n=3) were plotted against corresponding fold changes from microarray data (coefficient of determination, $r^2 = 0.97$).

Table I

Representative oligonucleotide primers used in quantitative real-time PCR

Gene Name	GenBank Accession No	Forward Primer	Reverse Primer
ATP-binding cassette, sub-family B (MDR/TAP),1A (Abcb1b)	NM_011075	5′-GAATGTCCAGTGGCTCCGA-3′	5′-CGGCTGTTGTCTCCATAGGC-3′
ATP-binding cassette, sub-family C (CFTR/MRP), 1 (Abcc1)	NM_008576	5′-CTCACGATTGCTCATCGGCT-3′	5′-AATCACCCGCGTGTAGTCCA-3′
CASP8 and FADD-like apoptosis regulator (Cflar)	NM_207653	5′-CCAGCTTTTCTTGTTTCCCAAG-3′	5′-CGGCGAACAATCTGGGTTAT-3′
Glutamate cysteine ligase, modifier subunit (Gclm)	NM_008129	5′-CGAGGAGCTTCGGGACTGTA-3′	5′-TGGTGCATTCCAAAACATCTG-3′
Glutathione S-transferase, alpha 4	NM_010357	5′-AGGAGTCATGGCAGCCAAAC-3′	5′-CCTCAAACTCCACTCCAGCC-3′
Glutathione S-transferase, mu3	NM_010359	5′-ATCCGCTTGCTCCTGGAATA-3′	5′-TTCTCACTCAGCCACTGGCTT-3′
Inhibitor of kappaB kinase gamma (Ikbkg)	NM_010547	5′-CTGAAAGTTGGCTGCCATGAG-3′	5′-GAGTGGTGAGCTGGAGCAGG-3′
Nuclear receptor coactivator 3 (Ncoa5)	NM-144892	5′-GAGGTGTCAGAGACGCCCAG-3′	5′-TTTCTTGTGGCCTTTGCTTTC-3′
Nuclear receptor interacting protein 1 (Nrip1)	NM_173440	5′-AACAGTGAGCTGCCAACCCT-3′	5′-CTTCGGGACCATGCAGATGT-3′
P300/CBP-associated factor (Pcaf)	NM_020005	5′-AGAGAGGCAGACAACGATCGA-3′	5′-TTGATGCGGTTCAGAAACATCT-3′
Protein kinase C, epsilon (Prkce)	NM_011104	5′-ACGCTCCTATCGGCTACGAC-3′	5′-CGAACTGGATGGTGCAGTTG-3′
Src family associated phosphoprotein 2 (Scap2)	NM_018773	5′- GCTGGCTACCTGGAAAAACG -3′	5′-TTCAAACCCCAGAAAGCTGTG-3′
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_008084	5′-CACCAACTGCTTAGCCCCC-3′	5′-TCTTCTGGGTGGCAGTGATG-3′

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Table II

TM-induced Nrf2-dependent genes in mouse small intestine and liver.

GenBank Accession	Gene Symbol	Gene Title	SIT*	Liver**
Cell Adhesion				
NM_009864	Cdh1	Cadherin 1	6.77	
XM_283264	Cdh10	cadherin 10		7.01
NM_007664	Cdh2	cadherin 2	9.72	
XM_488510	Cspg2	chondroitin sulfate proteoglycan 2	2.72	2.82
NM_009903	Cldn4	claudin 4	4.86	
NM_018777	Cldn6	claudin 6		2.32
NM_031174	Dscam	Down syndrome cell adhesion molecule (Dscam)	2.25	
NM_010103	Edil3	EGF-like repeats and discoidin I-like domains 3	9.2	
NM_008401	Itgam	integrin alpha M	2.42	
NM_008405	Itgb2l	integrin beta 2-like		9.64
	Jam3	Junction adhesion molecule 3	2.2	
NM_007736	Col4a5	procollagen, type IV, alpha 5	2.54	
XM_139187	Pcdh9	protocadherin 9		2.33
Apoptosis and Cell cycle control				
XM_194020	Acvr1c	activin A receptor, type IC	26.49	
NM_178655	Ank2	ankyrin 2, brain	17.4	
NM_153287	Axud1	AXIN1 up-regulated 1	2.71	
	Bcl2	B-cell leukemia/lymphoma 2 (Bcl2), transcript variant 1	2.68	
NM_009744	Bcl6	B-cell leukemia/lymphoma 6	2.02	
NM_207653	Cflar	CASP8 and FADD-like apoptosis regulator	2.12	
NM_026373	Cdk2ap2	CDK2-associated protein 2		2.35
XM_484088	Cdc27	cell division cycle 27 homolog (S. cerevisiae)	2.36	
NM_009862	Cdc45l	cell division cycle 45 homolog (S. cerevisiae)-like		3.38
NM_026201	Ccar1	cell division cycle and apoptosis regulator 1	9.84	
NM_013538	Cdca3	cell division cycle associated 3	2.18	
NM_011806	Dmtf1	cyclin D binding myb-like transcription factor 1	2.88	
NM_028399	Ccnt2	cyclin T2	9.26	
NM_009874	Cdk7	cyclin-dependent kinase 7 (homolog of Xenopus MO15 cdk-activating kinase)	15.94	
NM_007837	Ddit3	DNA-damage inducible transcript 3	13.72	9
NM_007950	Ereg	epiregulin	5.85	
NM_008087	Gas2	Growth arrest specific 2	2.01	
XM_137276	Gas2l3	growth arrest-specific 2 like 3	4.26	
NM_146071	Muc20	mucin 20		2.96
NM_009044	Rel	reticuloendotheliosis oncogene		2.35
NM_133810	Stk17b	serine/threonine kinase 17b (apoptosis-inducing)	2.27	
NM_028769	Syvn1	synovial apoptosis inhibitor 1, synoviolin		4.77
NM_021897	Trp53inp1	transformation related protein 53 inducible nuclear protein 1	2.49	
Biosynthesis and Metabolism				
	---	Acyl-CoA synthetase long-chain family member 5	17.14	
NM_029901	Akr1c21	aldo-keto reductase family 1, member C21		2.14
NM_023179	Atp6v1g2	ATPase, H+ transporting, V1 subunit G isoform 2		2.23
1451144_at	Bxdc2	brix domain containing 2	2.19	
NM_023525	Cad	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase		2.4
NM_198415	Ckmt2	creatine kinase, mitochondrial 2		29.85
NM_007710	Ckm	creatine kinase, muscle		21.08
NM_030225	Dlst	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	3.28	
NM_021896	Gucy1a3	guanylate cyclase 1, soluble, alpha 3	5.6	
NM_011846	Mmp17	matrix metallopeptidase 17	24.22	
NM_138656	Mvd	mevalonate (diphospho) decarboxylase		3.46
NM_009127	Scd1	stearoyl-Coenzyme A desaturase 1	2.26	
Calcium homeostasis				
NM_013471	Anxa4	Annexin A4	5.2	

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GenBank Accession	Gene Symbol	Gene Title	SIT*	Liver**
NM_009722	Atp2a2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2		2.65
NM_023116	Cacnb2	Calcium channel, voltage-dependent, beta 2 subunit	14.13	
NM_009781	Cacna1c	Calcium channel, voltage-dependent, L type, alpha 1C subunit	7.94	
NM_028231	Kcnmb2	potassium large conductance calcium-activated channel, subfamily M, beta member 2	4.62	
Cell Growth and Differentiation				
NM_010111	Efnb2	ephrin B2	2.67	
NM_177390	Myo1d	Myosin ID	2.68	
NM_145610	Ppan	peter pan homolog (Drosophila)		2.04
NM_021883	Tmod1	tropomodulin 1	2.7	
NM_009394	Tnnc2	troponin C2, fast		16.76
ER/Golgi transport and ER/Golgi biosynthesis/metabolism				
NM_025445	Arfgap3	ADP-ribosylation factor GTPase activating protein 3		2.58
NM_025505	Blzf1	basic leucine zipper nuclear factor 1	7.72	
NM_009938	Copa	coatomer protein complex subunit alpha		2.4
NM_025673	Golph3	Golgi phosphoprotein 3	2.57	
NM_146133	Golph3l	golgi phosphoprotein 3-like		2.41
NM_008408	Itm1	intergral membrane protein 1	6.62	2.56
NM_027400	Lman1	Lectin, mannose-binding		2.79
NM_025408	Phca	phytoceramidase, alkaline	3.04	
NM_009178	Siat4c	ST3 beta-galactoside alpha-2,3-sialyltransferase 4	2.4	
NM_020283	B3galt1	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 1		2.54
NM_011716	Wfs1	Wolfram syndrome 1 homolog (human)		2.02
Electron Transport				
NM_015751	Abce1	ATP-binding cassette, sub-family E (OABP), member 1		2.48
NM_010001	Cyp2c37	cytochrome P450, family 2. subfamily c, polypeptide 37	5.58	
NM_023913	Ern1	Endoplasmic reticulum (ER) to nucleus signalling 1		2.12
XM_129326	Gucy2g	guanylate cyclase 2g	2.39	
NM_007952	Pdia3	protein disulfide isomerase associated 3		3.11
NM_009787	Pdia4	protein disulfide isomerase associated 4		3.19
XM_907880	Pdia6	protein disulfide isomerase associated 6		2.9
XM_284053	Steap2	six transmembrane epithelial antigen of prostate 2	3.23	
NM_198295	730024F05Ri	Thioredoxin domain containing 10	2.91	
NM_029572	Txndc4	thioredoxin domain containing 4 (endoplasmic reticulum)		2.47
NM_023140	Txnl2	Thioredoxin-like 2	8.25	
G-protein coupled receptors				
NM_008158	Gpr27	G protein-coupled receptor 27	2.72	
NM_145066	Gpr85	G protein-coupled receptor 85		3.78
AK015353	Grm2	G protein-coupled receptor, family C, group 1, member B	2.18	
NM_008177	Grpr	gastrin releasing peptide receptor		2.18
NM_010314	Gngt1	guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 1		2.02
NM_139270	Pthr2	parathyroid hormone receptor 2		2.12
NM_011056	Pde4d	phosphodiesterase 4D, cAMP specific	2.78	
NM_022881	Rgs18	regulator of G-protein signaling 18		2.36
Kinases and Phosphatases				
NM_153066	Ak5	adenylate kinase 5	2.33	
NM_144817	Camk1g	calcium/calmodulin-dependent protein kinase I gamma		2.32
NM_139059	Csnk1d	Casein kinase 1, delta (Csnk1d), transcript variant 2	2.08	
NM_177914	MGI:3580254	diacylglycerol kinase kappa	13.2	
NM_130447	Dusp16	dual specificity phosphatase 16	3.17	2.13
NM_019987	Ick	intestinal cell kinase		2.06
XM_283179	Mast4	microtubule associated serine/threonine kinase family member 4	3.62	
NM_016700	Mapk8	mitogen activated protein kinase 8	12.43	
	Mapk8	mitogen activated protein kinase 8	7.41	
NM_172688	Map3k7	mitogen activated protein kinase kinase kinase 7	3.29	

GenBank Accession	Gene Symbol	Gene Title	SIT*	Liver**
NM_011101	Prkca	Protein kinase C, alpha	2.04	
NM_011104	Prkce	protein kinase C, epsilon	3.3	
NM_021880	Prkar1a	protein kinase, cAMP dependent regulatory, type I, alpha		15.23
NM_175638	Prkwnk4	Protein kinase, lysine deficient 4		8.16
NM_016979	Prkx	protein kinase, X-linked	2.27	
NM_133485	Ppp1r14c	Protein phosphatase 1, regulatory (inhibitor) subunit 14c		5.03
NM_012024	Ppp2r5e	protein phosphatase 2, regulatory subunit B (B56), epsilon isoform	2.49	
NM_008913	Ppp3ca	Protein phosphatase 3, catalytic subunit, alpha isoform	14.5	
AK134422	Ptp	Protein tyrosine phosphatase		3.27
NM_028259	Rps6kb1	ribosomal protein S6 kinase, polypeptide 1	2.05	
NM_031880	Tnk1	tyrosine kinase, non-receptor, 1	2.54	
Nuclear Assembly and Processing				
NM_010613	Khsrp	KH-type splicing regulatory protein	2.44	
NM_008671	Nap1l2	nucleosome assembly protein 1-like 2		4.7
NM_026175	Sf3a1	splicing factor 3a, subunit 1		2.77
NM_009408	Top1	Topoisomerase (DNA) I	5.94	
NM_008717	Zfml	Zinc finger, matrin-like		2.19
Glucose biosynthesis/metabolism				
NM_009605	Adipoq	adiponectin, C1Q and collagen domain containing		2.23
NM_018763	Chst2	Carbohydrate sulfotransferase 2		2.02
NM_008079	Galc	galactosylceramidase		2.95
NM_029626	Glt8d1	glycosyltransferase 8 domain containing 1		2.17
NM_013820	Hk2	hexokinase 2	2.46	
NM_010705	Lgals3	Lectin, galactose binding, soluble 3	3.2	
NM_199446	Phkb	phosphorylase kinase beta	2.06	
NM_016752	Slc35b1	solute carrier family 35, member B1		2.13
Signaling molecules and interacting partners				
NM_029291	Ascc2	Activating signal cointegrator 1 complex subunit 2		3.24
NM_007498	Atf3	activating transcription factor 3	8.73	
NM_016707	Bcl11a	B-cell CLL/lymphoma 11A (zinc finger protein)	4.2	
NM_033601	Bcl3	B-cell leukemia/lymphoma 3	2.08	
NM_007553	Bmp2	bone morphogenetic protein 2	2.55	
NM_007558	Bmp8a	bone morphogenetic protein 8a	2.39	
NM_178661	Creb3l2	cAMP responsive element binding protein 3-like 2		2.01
NM_010016	Daf1	decay accelerating factor 1	2.29	
NM_007897	Ebf1	early B-cell factor 1	8.98	
NM_023580	Epha1	Eph receptor A1		2.037
NM_133753	Errfi1	ERBB receptor feedback inhibitor 1	2.53	
NM_0010058	Erbb2ip	Erbb2 interacting protein	2.11	
NM_0010058	Erbb2ip	Erbb2 interacting protein	2.04	
NM_007906	Eef1a2	eukaryotic translation elongation factor 1 alpha 2		3.67
NM_007917	Eif4e	eukaryotic translation initiation factor 4E	3.05	
NM_173363	Eif5	eukaryotic translation initiation factor 5	2.13	
NM_010515	Igf2r	Insulin-like growth factor 2 receptor		2.22
NM_010591	Jun	Jun oncogene		2.29
NM_010592	Jund1	Jun proto-oncogene related gene d1		2.43
NM_008416	Junb	Jun-B oncogene	2.36	
NM_013602	Mt1	metallothionein 1		2.15
NM_008630	Mt2	metallothionein 2	2.79	
NM_170671	Mycbpap	Mycbp associated protein		3.97
NM_177619	Myst2	MYST histone acetyltransferase 2		2
NM_009123	Nkx1-2	NK1 transcription factor related, locus 2 (Drosophila)	2.45	
NM_030612	Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	2.67	
NM_017373	Nfil3	nuclear factor, interleukin 3, regulated	12.68	3.34
NM_144892	Ncoa5	nuclear receptor coactivator 5	14.65	
NM_173440	Nrip1	nuclear receptor interacting protein 1	2.87	

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BC032981	Nfxl1	nuclear transcription factor, X-box binding-like 1		3.13
NM_020005	Pcaf	P300/CBP-associated factor	2.33	
NM_027924	Pdgfd	platelet-derived growth factor, D polypeptide	6.17	
NM_017463	Pbx2	pre B-cell leukemia transcription factor 2		2.84
NM_026383	Pnrc2	proline-rich nuclear receptor coactivator 2		2.08
NM_145495	Rin1	Ras and Rab interactor 1	7.01	2.87
NM_011651	Stk22s1	serine/threonine kinase 22 substrate 1	2.36	
NM_175246	Snip1	Smad nuclear interacting protein 1		2.07
NM_007707	Socs3	suppressor of cytokine signaling 3	2.45	
NM_080843	Socs4	suppressor of cytokine signaling 4	2	
NM_009365	Tgfb1i1	transforming growth factor beta 1 induced transcript 1	2.22	
NM_0010130	Tgfbrap1	transforming growth factor, beta receptor associated protein 1	2.7	
NM_013869	Tnfrsf19	tumor necrosis factor receptor superfamily, member 19	2.4	
NM_010755	Maff	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	2.92	
NM_009524	Wnt5a	wingless-related MMTV integration site 5A		8.25
Transport				
NM_007511	Atp7b	ATPase, Cu++ transporting, beta polypeptide		2.34
NM_011075	Abcb1b	ATP-binding cassette, sub-family B (MDR/TAP), member 1B		4.65
NM_008576	Abcc1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	2.37	
NM_172621	Clic5	Chloride intracellular channel 5, mRNA		2.39
NM_024406	Fabp4	Fatty acid binding protein 4, adipocyte	3.7	
NM_146188	Kctd15	potassium channel tetramerisation domain containing 15	2.82	
	Kctd7	potassium channel tetramerisation domain containing 7		2.65
NM_148938	Slc1a3	solute carrier family 1 (glial high affinity glutamate transporter), member 3	4.03	
NM_019481	Slc13a1	solute carrier family 13 (sodium/sulphate symporters), member 1	2.09	
NM_0010041	Slc13a5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	6.88	
NM_011395	Slc22a3	solute carrier family 22 (organic cation transporter), member 3		2.07
NM_172980	Slc28a2	solute carrier family 28 (sodium-coupled nucleoside transporter), member 2	2	
NM_078484	Slc35a2	solute carrier family 35 (UDP-galactose transporter), member 2		6.5
NM_011990	Slc7a11	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	5.93	
NM_080852	Slc7a12	solute carrier family 7 (cationic amino acid transporter, y+ system), member 12		2.95
NM_011406	Slc8a1	Solute carrier family 8 (sodium/calcium exchanger), member 1	2.86	
NM_178892	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	2.27	
Ubiquitination and Proteolysis				
NM_027926	Cpa4	carboxypeptidase A4	2.29	
NM_011931	Cop1	Constitutive photomorphogenic protein		2.52
NM_013868	Hspb7	heat shock protein family, member 7 (cardiovascular)		2.25
NM_146042	Ibrdc2	IBR domain containing 2	6.77	
NM_009174	Siah2	seven in absentia 2	2.46	
	Siah2	seven in absentia 2		2.12
NM_025692	Ube1dc1	ubiquitin-activating enzyme E1-domain containing 1		2.79
NM_173443	Vcpip1	valosin containing protein (p97)/p47 complex interacting protein 1	2.12	
Molecular chaperones and Heat Shock Proteins				
NM_0010124	Hspb6	heat shock protein, alpha-crystallin-related, B6	2.13	
NM_010918	Nktr	natural killer tumor recognition sequence		2.02
NM_030201	Stch	stress 70 protein chaperone, microsome-associated, human homolog		2.47
	Stch	stress 70 protein chaperone, microsome-associated, human homolog	2.37	
Miscellaneous				
NM_008161	Gpx3	glutathione peroxidase 3		5.79
NM_028733	Pacsin3	Protein kinase C and casein kinase II substrate 3 (Pacsin3)		2.12
NM_009409	Top2b	Topoisomerase (DNA) II beta (Top2b), mRNA		3.39
NM_020283	B3galt1	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 1		2.45

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*Genes that were induced >2-fold by TM only in small intestine of Nrf2 wild-type mice but not in small intestine of Nrf2 knockout mice compared with vehicle treatment at 3h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.

**Genes that were induced >2-fold by TM only in liver of Nrf2 wild-type mice but not in liver of Nrf2 knockout mice compared with vehicle treatment at 3h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.

Table III

TM-suppressed Nrf2-dependent genes in mouse small intestine and liver.

GenBank Accession	Gene Symbo	Gene Title	SIT*	Liver**
Cell Adhesion				
NM _174988	Cdh22	cadherin 22	0.47	
NM_053096	Cml2	camello-like 2	0.45	
NM_009818	Catna1	Catenin (cadherin associated protein), alpha 1		0.13
NM_008729	Catnd2	Catenin (cadherin associated protein), delta 2		0.5
XM_488510	Cspg2	chondroitin sulfate proteoglycan 2	0.43	
NM_018764	Pcdh7	protocadherin 7	0.21	
NM_053134	Pcdhb9	protocadherin beta 9	0.28	
NM_033595	Pcdhga12	Protocadherin gamma subfamily A, 10, mRNA		0.4
Apoptosis and Cell cycle control				
NM_007566	Birc6	baculoviral IAP repeat-containing 6	0.49	
NM_009741	Bcl2	B-cell leukemia/lymphoma 2		0.25
NM_009950	Cradd	CASP2 and RIPK1 domain containing adaptor with death domain		0.43
NM_007609	Casp11	caspase 11, apoptosis-related cysteine peptidase	0.45	
NM_009811	Casp6	caspase 6	0.36	
NM_025680	Ctnnbl1	catenin, beta like 1	0.45	
NM_025866	Cdca7	cell division cycle associated 7		0.36
NM_026560	Cdca8	cell division cycle associated 8		0.37
XM_181420	Cgref1	cell growth regulator with EF hand domain 1	0.47	
NM_009131	Clec11a	C-type lectin domain family 11, member a	0.2	
NM_146207	Cul4a	cullin 4A	0.49	
NM_009873	Cdk6	cyclin-dependent kinase 6		0.47
NM_009876	Cdkn1c	cyclin-dependent kinase inhibitor 1C (P57)	0.48	
NM_007892	E2f5	E2F transcription factor 5	0.25	
NM_008655	Gadd45b	growth arrest and DNA-damage-inducible 45 beta		0.18
NM_183358	Gadd45gip1	growth arrest and DNA-damage-inducible, gamma interacting protein 1	0.45	
NM_010578	Itgb1	integrin beta 1 (fibronectin receptor beta)	0.12	
NM_019745	Pdcd10	programmed cell death 10	0.46	
NM_009383	Tial1	Tial1 cytotoxic granule-associated RNA binding protein-like 1	0.07	0.36
NM_009425	Tnfsf10	tumor necrosis factor (ligand) superfamily, member 10	0.33	
NM_009517	Wig1	wild-type p53-induced gene 1		0.5
Biosynthesis and Metabolism				
NM_177470	Acaa2	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	0.49	
NM_133904	Acacb	Acetyl-Coenzyme A carboxylase beta		0.14
NM_009695	Apoc2	apolipoprotein C-II	0.41	
NM_010174	Fabp3	Fatty acid binding protein 3, muscle and heart	0.23	
NM_008609	Mmp15	matrix metallopeptidase 15	0.46	
NM_023792	Pank1	pantothenate kinase 1	0.29	0.3
NM_144844	Pcca	propionyl-Coenzyme A carboxylase, alpha polypeptide	0.49	
NM_013743	Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4	0.4	
NM_019437	Rfk	riboflavin kinase	0.48	
NM_138758	Tmlhe	trimethyllysine hydroxylase, epsilon		0.37
NM_133995	Upb1	ureidopropionase, beta	0.42	
NM_009471	Umps	uridine monophosphate synthetase		0.36
Calcium homeostasis				
NM_013472	Anxa6	annexin A6		0.43
NM_007590	Calm3	calmodulin 3	0.43	
NM_023051	Clstn1	calsyntenin 1	0.5	
Electron Transport				
XM_485295	Cyb561d1	cytochrome b-561 domain containing 1	0.44	
NM_013809	Cyp2g1	cytochrome P450, family 2, subfamily g, polypeptide 1		0.43
NM_177380	Cyp3a44	cytochrome P450, family 3, subfamily a, polypeptide 44	0.39	
NM_018887	Cyp39a1	cytochrome P450, family 39, subfamily a, polypeptide 1	0.41	

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NM_010012	Cyp8b1	cytochrome P450, family 8, subfamily b, polypeptide 1		0.5
NM_170778	Dpyd	dihydropyrimidine dehydrogenase	0.49	
NM_010231	Fmo1	flavin containing monooxygenase 1	0.42	
NM_008631	Mt4	metallothionein 4	0.13	
NM_026614	Ndufa5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5	0.4	
NM_026610	Ndufb4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4	0.47	
NM_010887	Ndufs4	NADH dehydrogenase (ubiquinone) Fe-S protein 4	0.47	
NM_178239	Ndor1	NADPH dependent diflavin oxidoreductase 1		0.44
XM_128552	Pdia2	protein disulfide isomerase associated 2	0.43	
NM_025848	Sdhd	succinate dehydrogenase complex, subunit D, integral membrane protein	0.45	
NM_013711	Txnrd2	thioredoxin reductase 2		0.4
NM_011743	Zfp106	zinc finger protein 106	0.1	
Golgi assembly and glycosylation				
NM_007454	Ap1b1	adaptor protein complex AP-1, beta 1 subunit	0.49	
NM_028758	Gga2	Golgi associated, gamma adaptin ear containing, ARF binding protein 2		0.44
NM_008315	St3gal2	ST3 beta-galactoside alpha-2,3-sialyltransferase 2	0.5	
G-protein coupled receptors				
NM_008315	Htr7	5-hydroxytryptamine (serotonin) receptor 7 (Htr7), mRNA		0.47
NM_177231	Arrb1	arrestin, beta 1	0.38	
NM_030258	Gpr146	G protein-coupled receptor 146		0.49
NM_010309	Gnas	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	0.38	
NM_023121	Gngt2	guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2		0.47
NM_008142	Gnb1	guanine nucleotide binding protein, beta 1	0.5	
NM_053235	V1rc5	vomeronasal 1 receptor, C5		0.43
Kinases and Phosphatases				
NM_177343	Camk1d	Calcium/calmodulin-dependent protein kinase 1D		0.18
NM_009793	Camk4	Calcium/calmodulin-dependent protein kinase IV (Camk4)		0.11
NM_013642	Dusp1	dual specificity phosphatase 1		0.44
NM_010765	Mapkapk5	MAP kinase-activated protein kinase 5	0.47	
NM_011951	Mapk14	mitogen activated protein kinase 14	0.46	
NM_011944	Map2k7	mitogen activated protein kinase kinase 7		0.16
NM_023538	Mulk	multiple substrate lipid kinase	0.45	
NM_145962	Pank3	pantothenate kinase 3	0.4	
NM_00102495	Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	0.46	
NM_145401	Prkag2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	0.49	0.12
NM_017374	Ppp2cb	Protein phosphatase 2a, catalytic subunit, beta isoform	0.44	
NM_008914	Ppp3cb	protein phosphatase 3, catalytic subunit, beta isoform		0.44
NM_019651	Ptpn9	Protein tyrosine phosphatase, non-receptor type 9		0.23
NM_011213	Ptprf	protein tyrosine phosphatase, receptor type, F	0.4	
NM_009184	Ptk6	PTK6 protein tyrosine kinase 6	0.37	
NM_013845	Ror1	Receptor tyrosine kinase-like orphan receptor 1		0.43
NM_019924	Rps6ka4	ribosomal protein S6 kinase, polypeptide 4	0.43	
Nuclear assembly and processing				
NM_148948	Dicer1	Dicer1, Dcr-1 homolog (Drosophila)	0.43	
XM_131040	Hist2h2bb	Histone 2, H2bb	0.37	
NM_019786	Tbk1	TANK-binding kinase 1	0.4	
Glucose biosynthesis/metabolism				
NM_019395	Fbp1	fructose bisphosphatase 1	0.47	
NM_025799	Fuca2	fucosidase, alpha-L- 2, plasma	0.44	
NM_008155	Gpi1	glucose phosphate isomerase 1		0.49
NM_008061	G6pc	glucose-6-phosphatase, catalytic	0.26	
NM_00101337	Lman2l	lectin, mannose-binding 2-like		0.49
NM_008548	Man1a	mannosidase 1, alpha	0.45	
NM_010956	Ogdh	Oxoglutarate dehydrogenase (lipoamide)	0.25	
NM_00101336	Prkaa1	protein kinase, AMP-activated, alpha 1 catalytic subunit		0.49

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Signaling molecules and interacting partners				
NM_009755	Bmp1	bone morphogenetic protein 1	0.42	
NM_00101336	E2f8	E2F transcription factor 8		0.41
NM_010141	Epha7	Eph receptor A7		0.49
NM_020273	Gmeb1	glucocorticoid modulatory element binding protein 1	0.48	
NM_010323	Gnrhr	Gonadotropin releasing hormone receptor	0.28	
NM_176958	Hif1an	hypoxia-inducible factor 1, alpha subunit inhibitor	0.49	
NM_010547	Ikbkg	inhibitor of kappaB kinase gamma		0.45
NM_010515	Igf2r	insulin-like growth factor 2 receptor	0.44	
NM_010513	Igf1r	insulin-like growth factor I receptor	0.35	0.5
NM_009697	Nr2f2	Nuclear receptor subfamily 2, group F, member 2		0.45
	Pdap1	PDGFA associated protein 1		0.47
NM_013634	Pparbp	peroxisome proliferator activated receptor binding protein	0.44	
NM_027230	Prkcbp1	protein kinase C binding protein 1	0.48	
NM_026880	Pink1	PTEN induced putative kinase 1	0.48	
NM_018773	Scap2	src family associated phosphoprotein 2	0.49	
NM_007706	Socs2	Suppressor of cytokine signaling 2		0.44
NM_178111	Trp53inp2	tumor protein p53 inducible nuclear protein 2		0.47
NM_011703	Vipr1	vasoactive intestinal peptide receptor 1		0.4
NM_010153	Erbb3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	0.46	
Transport				
NM_009727	Atp8a1	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1		0.44
NM_024173	Atp6v1g1	ATPase, H+ transporting, V1 subunit G isoform 1	0.46	
NM_029600	Abcc3	Multidrug resistance-associated protein 3 (Abcc3)		0.46
NM_010604	Kcnj16	potassium inwardly-rectifying channel, subfamily J, member 16	0.15	
NM_018824	Slc23a2	Sodium-dependent vitamin C transporter type 2 (Slc23a1)		0.45
NM_011397	Slc23a1	solute carrier family 23 (nucleobase transporters), member 1		0.3
NM_008063	Slc37a4	solute carrier family 37 (glycerol-6-phosphate transporter), member 4	0.46	
NM_018760	Slc4a4	solute carrier family 4 (anion exchanger), member 4	0.5	
NM_016917	Slc40a1	Solute carrier family 40 (iron-regulated transporter), member 1		0.45
XM_127434	Slc9a3	solute carrier family 9 (sodium/hydrogen exchanger), member 3	0.41	
Detoxifying enzymes				
NM_008129	Gclm	glutamate-cysteine ligase , modifier subunit	0.45	
NM_029555	Gstk1	glutathione S-transferase kappa 1	0.45	
NM_010357	Gsta4	glutathione S-transferase, alpha 4	0.49	
NM_010359	Gstm3	glutathione S-transferase, mu 3	0.22	
XM_359308	Gstm7	glutathione S-transferase, mu 7	0.46	
NM_008185	Gstt1	glutathione S-transferase, theta 1	0.43	
NM_025304	Lcmt1	leucine carboxyl methyltransferase 1	0.48	
NM_019946	Mgst1	microsomal glutathione S-transferase 1	0.18	
NM_025569	Mgst3	microsomal glutathione S-transferase 3	0.47	
NM_019878	Sult1b1	sulfotransferase family 1B, member 1	0.41	
Ubiquitination and Proteolysis				
NM_011780	Adam23	A disintegrin and metallopeptidase domain 23		0.34
NM_007754	Cpd	carboxypeptidase D	0.45	
NM_134015	Fbxw11	F-box and WD-40 domain protein 11	0.27	
NM_177703	Fbxw19	F-box and WD-40 domain protein 19		0.11
NM_028705	Herc3	hect domain and RLD 3		0.35
NM_145486	Mar 2	membrane-associated ring finger (C3HC4) 2	0.5	0.28
NM_020487	Prss21	protease, serine, 21	0.12	
NM_008944	Psma2	proteasome (prosome, macropain) subunit, alpha type 2	0.49	
NM_013640	Psmb10	proteasome (prosome, macropain) subunit, beta type 10	0.5	
XM_483996	Usp34	ubiquitin specific peptidase 34		0.49
NM_013918	Usp25	Ubiquitin-specific processing protease		0.47
Molecular Chaperones and Heat Shock Proteins				
NM_146036	Ahsa1	AHA1, activator of heat shock 90kDa protein ATPase homolog 1 (yeast)		0.4

GenBank Accession	Gene Symbo	Gene Title	SIT*	Liver**
NM_025384	Dnajc15	DnaJ (Hsp40) homolog, subfamily C, member 15	0.5	
NM_139139	Dnajc17	DnaJ (Hsp40) homolog, subfamily C, member 17		0.43
NM_024219	Hsbp1	heat shock factor binding protein 1	0.46	
	Hspa1b	heat shock protein 1B		0.41
NM_019960	Hspb3	heat shock protein 3	0.3	
Miscellaneous				
NM_008708	Nmt2	N-myristoyltransferase 2	0.14	
NM_007453	Prdx6	peroxiredoxin 6	0.46	
NM_011434	Sod1	Superoxide dismutase 1, soluble	0.25	

*Genes that were suppressed >2-fold by TM only in small intestine of Nrf2 wild-type mice but not in small intestine of Nrf2 knockout mice compared with vehicle treatment at 3h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.

**Genes that were suppressed >2-fold by TM only in liver of Nrf2 wild-type mice but not in liver of Nrf2 knockout mice compared with vehicle treatment at 3h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.