A Biochemical, Toxicological And Proteomic Analysis Investigating The Effect Of Nrf2 Gene Deletion On Paracetamol- Induced Hepatotoxicity *In Vivo*.

Laura Randle[1] (lrandle@liv.ac.uk), Neil Kitteringham[1], Rowena Sison[1], Daniela Denk[2], Roz Jenkins[1], Daniel Antoine[1], Joanne Henry[1], Luke Palmer[1], Vivian Platt[1], Valerie Tilston[1], Dominic Williams[1], Anja Kipar[2], John Hayes[3], Masayuki Yamamoto[4], Chris Goldring[1] and Kevin Park[1].

[1] Department of Pharmacology & Therapeutics, University of Liverpool, U.K. [2] Department of Veterinary Pathology, University of Liverpool, U.K. [3] Biomedical Research Institute, Ninewells Hospital, University of Dundee, U.K. [4] Department of Medicinal Chemistry, Tohoku University Graduate School of Medicine, Japan.

1 Introduction

- The ability of a cell to rapidly adapt to its environment is essential for survival. The Nrf2-Keap1 signalling pathway enables adaptation to oxidative and electrophilic stress by stimulating the transcriptional activation of ~ 100 cytoprotective genes to restore cellular homeostasis [1]. This is achieved by increasing cellular thiols, limiting free radical production and up-regulating drug metabolising enzymes and transporters to assist detoxification.
- We have previously reported that Paracetamol (APAP) can induce Nrf2 translocation and defence gene expression *in vivo* and *in vitro*, in a dose-dependent manner [2, 3]. Nrf2 gene deletion studies have demonstrated that null mice are more susceptible to xenobiotic toxicity in a variety of tissues [4-6].
- Toxicoproteomics seeks to identify critical proteins and pathways that are affected by and respond to adverse chemical and environmental exposures by combining traditional toxicology, differential global protein and gene expression analysis, and systems biology [7,8], enabling an unbiased quantitative analysis of global proteome expression.
- Isobaric tagging for relative quantification (iTRAQ) has allowed us to track the influence of Nrf2 gene deletion on APAP-induced hepatotoxicity *in vivo*. Currently there are no global hepatic proteomic studies exploring Nrf2 null mice. Many studies have concentrated on investigating Nrf2-dependent changes at the transcriptomic level; however these may not necessarily be translated into changes in protein level and function.

2 Aim

- Our aim is to understand the temporal and dose-dependent profile of Nrf2 -mediated adaptation to cellular stress.
- We are investigating the consequence of Nrf2 gene deletion on the initiation and progression of APAP-induced hepatotoxicity, to establish an Nrf2 dependent protein signature set to inform future drug discovery and development through understanding the early molecular events that occur in drug -induced liver injury (DILI).
- Clinical chemistry and histopathological changes can facilitate the 'phenotypic anchoring' of molecular expression data, providing a biological context for toxicoproteomic observations at sub -toxic APAP doses.

3 Methods

- **Animal model:** An Nrf2 KO colony was established at the University of Liverpool [9]. Fed male littermate C57BL6J Nrf2 WT and KO mice (10-12wks) received a single *i.p.* injection of APAP (300 mg/kg) or saline control at 0, 1, 3 & 5h (n=6/ group, t=10am).
- **Hepatotoxicity:** Serum ALT and HMGB1 levels were determined as indicators of hepatocellular injury [10].
- **Histology:** Liver sections were stained with haematoxylin eosin and evaluated independently by two co-authors (blind), applying a pre-defined scoring system for the degree of hepatocellular necrosis [grade 0 (none) to 5 (severe)].
- **Hepatic biochemicals:** Reduced GSH levels were determined via a DTNB-GSSG recycling assay [11]. Ophthalmic acid (OA) levels were determined using LC-MS/MS with MRM analysis (m/z transition 290.4/58.0) on a API2000. OA is a GSH analogue requiring Nrf2 dependent biosynthetic consecutive reactions of glutamate cysteine ligase (GCLC) and glutathione synthetase (GS) *in vivo* (Fig. 1) [12].

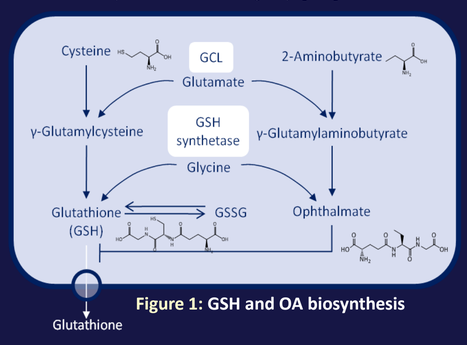


Figure 1: GSH and OA biosynthesis

- **Quantitative Real Time PCR Analysis:** Fifteen Nrf2 dependent genes were analysed in (n=6) using SYBR green QRT-PCR and normalised to housekeeping genes. Data from pooled 1h treatment groups is expressed as mean hepatic fold change from 3 individual experiments.
- **Multiplex Proteomics:** Global hepatic protein expression was assessed at 3h using 8-plex iTRAQ labelling according to the manufacturer's instructions, subjected to strong cation exchange (CEX) and LC-MS/MS analysis (QSTAR Pulsar system). Proteins were identified using *protein pilot 3* software with *Panther* classification system (v2.5). Each 8-plex contained 2 mice per treatment group. Mean expression was calculated for WT control, WT APAP, KO control and KO APAP groups (n=6/group). Only proteins identified with >90% confidence and ≥ 2 peptides and a False Discovery Rate <1% were included in analysis. Mean ratios were calculated between treatment. Proteins with mean expression levels < or > 1 SD from the global WT control mean were included in further group comparisons.

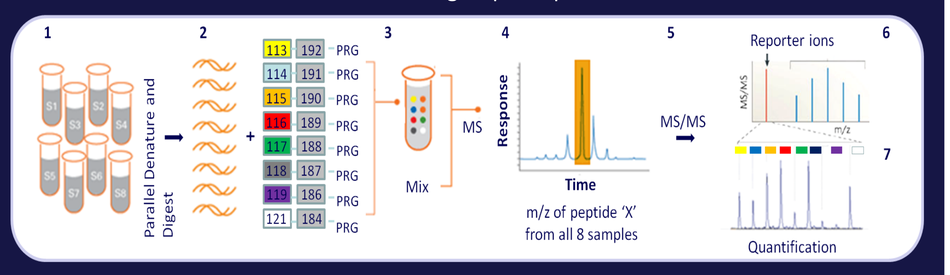


Figure 2: iTRAQ labelling summary. 1. 75ug protein/ mouse **2.** Tryptic digest and iTRAQ Labelling **3.** Pool – CEX **4.** LC-MS: select specific peptide **5.** LC-MS/MS: peptide fragmentation **6.** Identification: peptide sequencing **7.** Relative quantification of each reporter ions

- **Statistical Analysis**: Results are expressed as mean SEM and were analysed for non-normality using a Shapiro-Wilk test. Normally distributed data: Student's unpaired t test or ANOVA with Tukey multiple comparison test. Non-normally distributed data: Mann Whitney U test.

4 Results: A. Histopathology and clinical chemistry for phenotypic anchoring

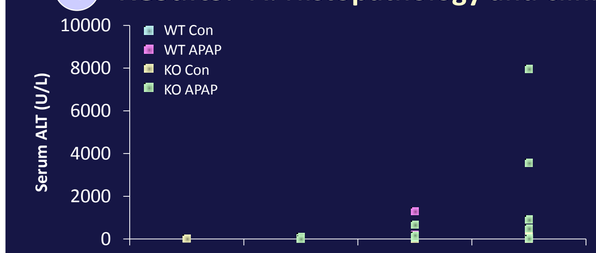


Figure 3: Mean Serum ALT levels in Nrf2 WT and KO mice at 0-5h post APAP treatment

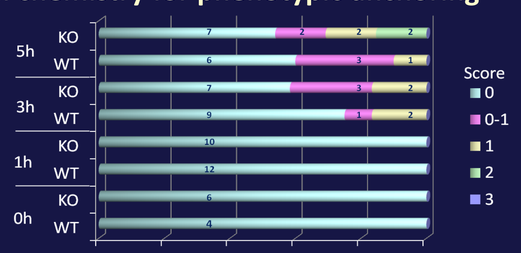


Figure 4: Summary of histological changes in Nrf2 WT and KO mice at 0-5hr post APAP treatment

- There was an upward trend in mean serum ALT levels after sub-toxic APAP treatment over the 5h time course. This was, however not significantly different (Fig. 3). Serum ALTs correlated with serum HMBG1 levels (R^2 = 0.7853) over time indicating passive release following hepatocellular injury.
- No histological changes (score 0) were observed in the liver of untreated WT and KO mice at 0h, APAP treated mice after 1h, and in all vehicle controls at each time point (Fig. 4).
- At 3h post APAP, mild hepatocellular damage was seen in both WT and KO mice. At 5h, the damage had on average intensified in the KO mice (Fig. 4), indicating a higher sensitivity to APAP toxicity.

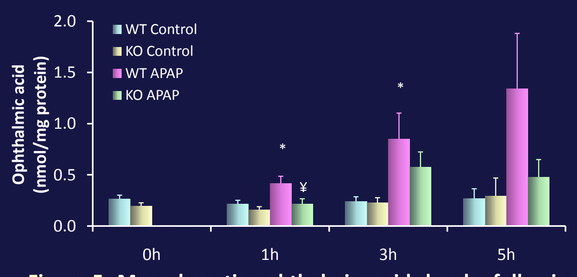


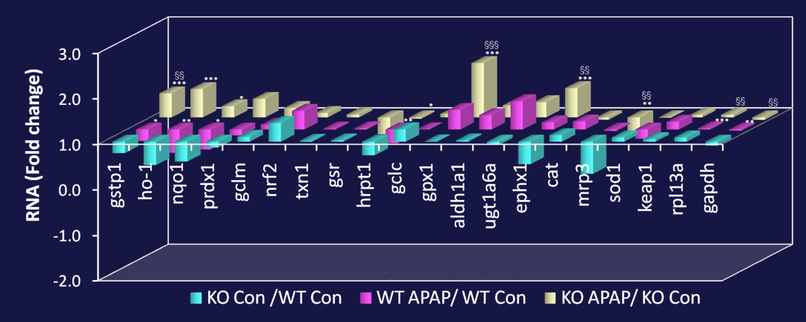
Figure 5: Mean hepatic ophthalmic acid levels following APAP treatment (0-5h) in Nrf2 WT and KO mice.

*P<0.05; ** P<0.01; ***P<0.001 *vs.* time matched WT saline control. P<0.05; P<0.01; P<0.001 *vs.* time matched KO saline control; ¥¥P<0.01 *vs.* time matched WT APAP control.

- APAP significantly depleted hepatic GSH levels in WT and KO treated mice within 1h, indicative of bioactivation. GSH levels were replenished in WT mice over the duration of the time course but not in APAP treated KO mice (data not shown).
- Whilst hepatic OA was significantly induced in the WT animals after APAP treatment, this increase was (significantly) less in the KO animals, as expected from the role of Nrf2 in GCLC and GS regulation (Fig. 5).

4 B. Nrf2 Hepatic Transcriptomic and Proteomic Profile

Figure 6: Mean Nrf2 dependent gene expression in Nrf2 WT and KO mice at 1h.



- gstp1, ho-1, nqo1, ephx1 and mrp3 gene expression was lower in Nrf2 KO mice at 1h (Fig. 6).
- Observed changes in APAP treated KO mice within ephx1, gpx1 and ho-1 genes were Nrf2 independent.

*P<0.05, **P<0.01, ***P<0.001 vs. KO Con/ WT Con and P<0.01, P<0.001 vs. WT APAP/ WT Con

Figure 7: A heat map to illustrate basally regulated Nrf2-dependent protein expression at 3h.

Accession	Gene ID	Name	KO Control / WT Control
Q05816	FABP5	Fatty acid-binding protein, epidermal	
Q8VBT2	SDHL	L-serine dehydratase	
P16460	ASSY	Argininosuccinate synthase	
P06151	LDHA	L-lactate dehydrogenase A chain	
Q921I1	TRFE	Serotransferrin	
Q8C196	CPSM	Carbamoyl-phosphate synthase [ammonia], mitochondrial	
P52840	ST1A1	Sulfotransferase 1A1	
P19096	FAS	Fatty acid synthase	
P32020	NLTP	Non-specific lipid-transfer protein	
Q9QXD6	F16P1	Fructose-1,6-bisphosphatase 1	
Q61207	SAP	Sulfated glycoprotein 1	
Q91V92	ACLY	ATP-citrate synthase	
P25688	URIC	Uricase	
Q8VCH0	THIKB	3-ketoacyl-CoA thiolase B, peroxisomal	
Q9DBE0	CSAD	Cysteine sulfinic acid decarboxylase	
P84244	H33	Histone H3.3	
P12710	*FABPL*	*Fatty acid-binding protein, liver*	
Q00898	A1AT5	Alpha-1-antitrypsin 1-5	
Q63880	*EST31*	*Liver carboxylesterase 31*	
P60710	*ACTB*	*Actin, cytoplasmic 1*	
Q8VDD5	MYH9	Myosin-9	
P21981	TGM2	Protein-glutamine gamma-glutamyltransferase 2	
Q9R0H0	ACOX1	Peroxisomal acyl-coenzyme A oxidase 1	
P14211	CALR	Calreticulin	
P05202	AATM	Aspartate aminotransferase, mitochondrial	
Q8JZR0	ACSL5	Long-chain-fatty-acid--CoA ligase 5	
P35492	HUTH	Histidine ammonia-lyase	
Q8BWT1	THIM	3-ketoacyl-CoA thiolase, mitochondrial	
Q91X83	METK1	S-adenosylmethionine synthetase isoform type-1	
P31786	ACBP	Acyl-CoA-binding protein	
P20029	GRP78	78 kDa glucose-regulated protein	
Q99P30	NUDT7	Peroxisomal coenzyme A diphosphatase NUDT7	
P58252	EF2	Elongation factor 2	
P40936	INMT	Indolethylamine N-methyltransferase	
Q920E5	FPPS	Farnesyl pyrophosphate synthetase	
P63158	HMGB1	High mobility group protein B1	
Q91X72	HEMO	Hemopexin	
Q91X91	NADC	Nicotinate-nucleotide pyrophosphorylase [carboxylating]	
P09103	PDIA1	Protein disulfide-isomerase	
Q9DBA8	HUTI	Probable imidazolonepropionase	
P18242	CATD	Cathepsin D	
Q921X9	PDIA5	Protein disulfide-isomerase A5	
P61979	HNRPK	Heterogeneous nuclear ribonucleoprotein K	
P05201	AATC	Aspartate aminotransferase, cytoplasmic	
P35505	FAAA	Fumarylacetoacetase	
Q8VCR2	DHB13	17-beta hydroxysteroid dehydrogenase 13	
O35490	BHMT1	Betaine--homocysteine S-methyltransferase 1	
P08003	PDIA4	Protein disulfide-isomerase A4	
Q64727	VINC	Vinculin	
P27773	PDIA3	Protein disulfide-isomerase A3	
P97872	*FMO5*	*Dimethylaniline monooxygenase [N-oxide-forming] 5*	
Q99LX0	*PARK7*	*Protein DJ-1*	
Q60605	MYL6	Myosin light polypeptide 6	
P49429	HPPD	4-hydroxyphenylpyruvate dioxygenase	
Q8K0E8	FIBB	Fibrinogen beta chain	
Q00623	APOA1	Apolipoprotein A-I	
P62806	H4	Histone H4	
P24369	PPIB	Peptidyl-prolyl cis-trans isomerase B	
Q8CAY6	THIC	Acetyl-CoA acetyltransferase, cytosolic	
P00920	CAH2	Carbonic anhydrase 2	
P62991	UBIQ	Ubiquitin	
Q9DCX2	ATP5H	ATP synthase subunit d, mitochondrial	
P67778	PHB	Prohibitin	
Q61838	A2M	Alpha-2-macroglobulin	
Q8VCM7	FIBG	Fibrinogen gamma chain	
P10126	EF1A1	Elongation factor 1-alpha 1	
P62897	CYC	Cytochrome c, somatic	
P01942	HBA	Hemoglobin subunit alpha	
Q01853	*TERA*	*Transitional endoplasmic reticulum ATPase*	
P38647	GRP75	Stress-70 protein, mitochondrial	
P00329	ADH1	Alcohol dehydrogenase 1	

Accession	Gene ID	Name	KO Control / WT Control
P10649	GSTM1	Glutathione S-transferase Mu 1	
P17717	UD2B5	UDP-glucuronosyltransferase 2B5	
P19157	GSTP1	Glutathione S-transferase P 1	
Q91X77	CY250	Cytochrome P450 2C50	
Q9D379	HYEP	Epoxide hydrolase 1	
P02762	MUP6	Major urinary protein 6	
Q64442	DHSO	Sorbitol dehydrogenase	
P06801	*MAOX*	*NADP-dependent malic enzyme*	
P30115	GSTA3	Glutathione S-transferase A3	
Q8VC30	DHAK	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	
Q8VCC2	EST1	Liver carboxylesterase 1	
Q91VA0	ACSM1	Acyl-coenzyme A synthetase ACSM1, mitochondrial	
P24549	AL1A1	Retinal dehydrogenase 1	
P47738	ALDH2	Aldehyde dehydrogenase, mitochondrial	
Q8VCW8	ACSF2	Acyl-CoA synthetase family member 2, mitochondrial	
O70475	UGDH	UDP-glucose 6-dehydrogenase	
Q9ET01	PYGL	Glycogen phosphorylase, liver form	
Q64458	CP2CT	Cytochrome P450 2C29	
O08709	PRDX6	Peroxiredoxin-6	
Q9DBG1	CP27A	Cytochrome P450 27, mitochondrial	
P15105	GLNA	Glutamine synthetase	
P15626	GSTM2	Glutathione S-transferase Mu 2	
Q9JII6	AK1A1	Alcohol dehydrogenase [NADP+]	
Q91VS7	MGST1	Microsomal glutathione S-transferase 1	
O55022	PGRC1	Membrane-associated progesterone receptor component 1	
Q9EQ20	MMSA	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	
Q63836	SBP2	Selenium-binding protein 2	
Q8R0Y6	FTHFD	10-formyltetrahydrofolate dehydrogenase	
P53657	KPYR	Pyruvate kinase isozymes R/L	
P11589	MUP2	Major urinary protein 2	
Q05421	CP2E1	Cytochrome P450 2E1	
P24472	GSTA4	Glutathione S-transferase A4	
Q91VD9	NDUS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	
P16331	PH4H	Phenylalanine-4-hydroxylase	
P70694	DHB5	Estradiol 17 beta-dehydrogenase 5	
P97328	KHK	Ketohexokinase	

≤2 0 ≥2 Fold change

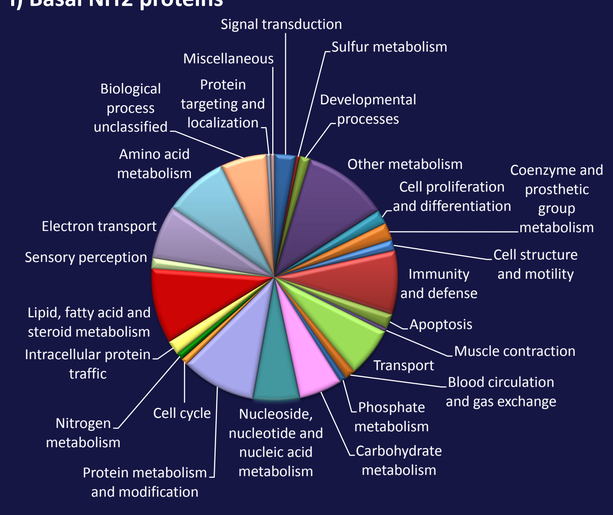
Figure 8: A heat map to illustrate Nrf2-regulated APAP responsive protein expression at 3h.

Accession	Gene ID	Name	WT APAP / WT Control
Q99LX0	*PARK7*	*Protein DJ-1*	
Q63880	*EST31*	*Liver carboxylesterase 31*	
Q61316	HSP74	Heat shock 70 kDa protein 4	
P06801	*MAOX*	*NADP-dependent malic enzyme*	
P62082	RS7	40S ribosomal protein S7	
Q8R086	SUOX	Sulfite oxidase, mitochondrial	
Q01853	*TERA*	*Transitional endoplasmic reticulum ATPase*	
Q03265	ATPA	ATP synthase subunit alpha, mitochondrial	
Q9R1J0	NSDHL	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	
P12710	*FABPL*	*Fatty acid-binding protein, liver*	
Q8BG05	ROA3	Heterogeneous nuclear ribonucleoprotein A3	
P11725	OTC	Ornithine carbamoyltransferase, mitochondrial	
Q00897	A1AT4	Alpha-1-antitrypsin 1-4	
P60335	PCBP1	Poly(rC)-binding protein 1	
P60710	*ACTB*	*Actin, cytoplasmic 1*	
P33267	CP2F2	Cytochrome P450 2F2	
P97872	*FMO5*	*Dimethylaniline monooxygenase [N-oxide-forming] 5*	
Q8CIM7	CP2DQ	Cytochrome P450 2D26	
Q6ZWV3	RL10	60S ribosomal protein L10	
P21614	VTDB	Vitamin D-binding protein	

- 452 proteins were identified in ≥ 4mice within each treatment group. 107 proteins were under Nrf2 basal regulation (Fig. 7), 20 were Nrf2 dependent and responsive to APAP at 3h (Fig. 8) and 7 proteins were both *basally regulated and APAP responsive*.
- Several proteins were found to have a higher basal expression in KO mice, this may represent compensatory changes to cope with an impaired defence response.

C. Biological processes which are regulated by Nrf2 dependent proteins

i) Basal Nrf2 proteins



ii) Nrf2 dependent, APAP responsive proteins

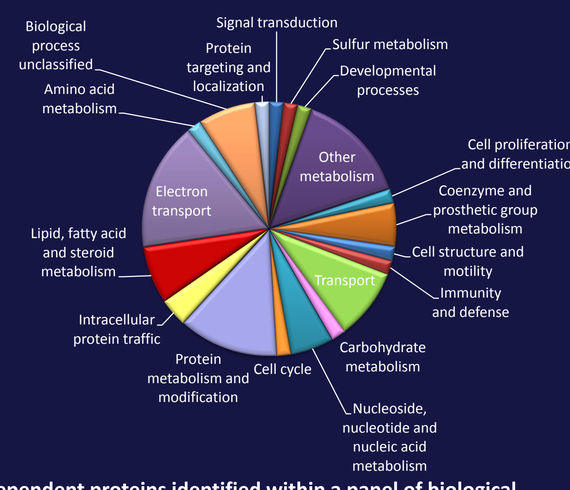


Figure 9: Panther pie charts to illustrate the proportion of Nrf2 dependent proteins identified within a panel of biological processes under basal (i) and APAP-treated conditions (ii).

- Nrf2 dependent proteins are involved in a wide variety of biological processes (Fig. 9).
- Following 3h APAP treatment the electron transport, protein metabolism/modification and coenzyme metabolism processes increase at the expense of amino acid and lipid metabolism as the cell initiates cellular defence mechanisms to overcome the sub-toxic insult.

5 Conclusions

- KO mice are more sensitive to APAP toxicity.
- Nrf2 regulates a specific suite of proteins to facilitate a coordinated response to an acute APAP insult.
- Some previously-reported Nrf2-dependent proteins are absent from the iTRAQ analysis. This may be because small changes below our threshold cut-off have not been included in the initial group analysis shown here. Our data requires further interrogation at the level of individual mouse data to anchor these changes to their toxicological and biochemical phenotype, which may uncover further subtle differences in protein expression.

6 References

[1] Hayes *et al.*, TiBS. 2009; 34 (4), 176-188. **[2]** Goldring *et al.*, Hepatology. 2004; 39 (5):1267- 1276. **[3]** Copple *et al.*, Hepatology. 2009; 48(4):1292-1301. **[4]** Enomoto *et al.*, Toxicol. Sci. 2001; 59, 169-177. **[5]** Sriram *et al.*, Pulm. Pharmacol. Ther. 2009; 22 (3):221-236. **[6]** Park et al., Biochem Pharmacol. 2008; 76(5): 597-607. **[7]** Wetmore *et al.*, Tox Pathol. 2004; 32, 619-642. **[8]** Waters *et al.*, Nat. Gen. Rev. 2009; 5, 936-948. **[10]** Antoine et al., Expert Op. Drug Metab. Toxicol. 2008; 4 (11) 1415-1427. **[11]** Vandeputte *et al.*, Cell Biol Toxicol. 1994; 10 (5-6): 415-21. **[12]** Soga *et al.*, JBC. 2006; 281 (24) 16768-16776.