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Dual roles for splice variants of the glucuronidation pathway as regulators of cellular metabolism.

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Abbreviations: UGT, UDP-glucuronosyltransferases; i1, isoform 1; i2, isoform 2; co-IP, co-immunoprecipitation; SN-38, 7-Ethyl-10-hydroxycamptothecin; CAT, catalase; Prdx1, Peroxiredoxin 1; ER, endoplasmic reticulum; GI, gastrointestinal; KD, knockdown; ROS, reactive oxygen species; HBA, hemoglobin alpha; SOD1, superoxide dismutase 1; SILAC, stable isotope labeling of amino acids in cell culture; MS, mass spectrometry.

ABSTRACT

Transcripts of the *UGT1A* gene, encoding half of human UGT enzymes, undergo alternative splicing, resulting in active enzymes named isoforms 1 (i1s) and novel truncated isoforms 2 (i2s). Here, we investigated the effects of depleting endogenous i2 on drug response and to unveil any additional biological role(s) for the truncated novel UGT proteins. We used an integrated systems biology approach that combines RNA interference with unbiased global genomic and proteomic screens, and used the HT115 colorectal cancer cells as a model. Consistent with previous evidence suggesting that i2s negatively regulate i1s through protein-protein interactions, i2-depleted cells were less sensitive to drug-induced cell death (IC_{50} of $0.45 \pm 0.05 \mu\text{M}$ vs. $0.22 \pm 0.03 \mu\text{M}$; $P=0.006$), demonstrating that modulation of i2 levels meaningfully impacts drug bioavailability and cellular response. We also observed reduced production of reactive oxygen species by 30 % ($P<0.05$), and an enhanced expression (>1.2 fold; $P<0.05$) of several proteins such as hemoglobin alpha genes and superoxide dismutase 1 that have network functions associated with antioxidant properties. Interaction proteomics analysis of endogenous proteins from the cellular model, mainly in human intestine but also in kidney tissues further uncovered interactions between i2s (but not i1s) and the antioxidant enzymes catalase and peroxiredoxin 1, which may influence antioxidant potential through sequestration of these novel partners. Our findings demonstrate for the first time dual roles for i2s in the cellular defense system as endogenous regulators of drug response as well as in oxidative stress.

INTRODUCTION

Conjugative cellular metabolism mediated by UDP-glucuronosyltransferases (UGTs) has a clear effect on xenobiotics and endobiotics bioavailability as well as an influence on drug response, treatment outcomes, and disease susceptibility and progression (Guillemette et al., 2010). Alternative splicing is one of the most significant components of the functional complexity of human UGTs. *UGT1A* is an ideal yet complex example of this protein repertoire-expanding mechanism because it gives rise to at least 27 unique mRNAs and 18 functional proteins (Bellemare et al., 2010b). These numerous alternative UGT1A transcripts are created by a series of splicing events involving 13 alternative first exons (1A1 through 1A13) and alternative terminal fifth exons (5a and 5b) (Girard et al., 2007). The alternative usage of first exons and the terminal exon 5a lead to classical variant mRNAs (v1) encoding nine enzymatically active UGT1A enzymes (isoforms 1, or i1) with distinct substrate specificity and tissue expression profile. The inclusion of the terminal exon 5b yields nine distinct UGT1A_v2 mRNAs (containing exon 5b but not 5a) and nine distinct UGT1A_v3 mRNAs (containing exons 5a and 5b), each having an identical open reading frame encoding nine shorter polypeptides named UGT1A isoforms 2 (i2).

The i2 and i1 proteins have similar expression patterns in human tissues, are highly abundant in the gastrointestinal (GI) tract and co-localize in the endoplasmic reticulum (ER), but i2 are devoid of transferase activity (Levesque et al., 2007; Bellemare et al., 2011). Further functional assays with human cells co-expressing both i1s and i2s clearly demonstrated a significant decrease in cellular glucuronidation activity for many different UGT1A substrates (Girard et al., 2007; Bellemare et al., 2010b). It was previously shown that transient repression of i2 by small interfering RNAs leads to an increased glucuronidation activity in human colon and liver cells

revealing a novel regulatory mechanism by i2s (Bellemare et al., 2010c; Jones et al., 2012). UGT oligomerization is likely the underlying mechanism for the negative modulation of transferase activity by splice variant i2. Indeed, several groups demonstrated that UGTs act as dimers/tetramers (Fujiwara et al., 2007; Operana et al., 2007), and experimental evidence demonstrated a direct interaction between i1-i2 proteins (Bellemare et al., 2010a; Bellemare et al., 2010b).

We proposed that the novel i2 splice products may be biologically relevant with physiological and pharmacological influences based on the following: 1) their wide expression profile (Girard et al., 2007; Bellemare et al., 2011); 2) their substantial expression levels particularly in the GI tract and kidneys (Girard et al., 2007; Levesque et al., 2007; Bellemare et al., 2011); 3) their often concurrent expression with the UGT1A_{i1} enzymes in the same healthy tissue structures and cell types (Bellemare et al., 2011); 4) an evident lack of correlation between mRNA expression levels, UGT1A protein expression and transferase activity (Ohtsuki et al., 2012), which might be explained by i2 alternative splicing events recently reported (Girard et al., 2007; Levesque et al., 2007); 5) the modulatory function of i2 on cellular UGT activity *in vitro* that is comparable to the effect observed for genetic polymorphisms having an influence on drug response *in vivo* (Iyer et al., 2002); and finally, 6) the observation that i2s do not reside exclusively in the ER (where i1s localize) and in fact can be detected in the cytoplasm (Levesque et al., 2007), raising the possibility that i2s may have additional cellular functions perhaps independent of their role in drug metabolism.

To test this hypothesis, we developed a stable knockdown (KD) model of i2s using short hairpin RNA (shRNA) in HT115 colon cancer cells, which express both classes of i1 and i2 with almost equivalent level of v2/v3 transcripts encoding i2 isoforms relative to v1 levels encoding UGT1A

enzymes (0.8:1) (Bellemare et al., 2010c). We used, as a pharmacological model compound, the active metabolite of irinotecan (CPT-11), namely SN-38, which is almost exclusively metabolized by the UGT1A pathway and is one of the most common cytotoxic agents used to treat metastatic colorectal cancer. The interindividual variability of the UGT1A pathway induced by genetic variants such as the UGT1A1*28 allele has well-defined clinical relevance by considerably affecting the glucuronidation of SN-38 and risk for drug-induced adverse effects in cancer patients (Iyer et al., 2002). Using novel and unbiased approaches we have studied the impact of i2's depletion on cell metabolism and demonstrated that KD of i2 increases drug metabolism and decreases colon cancer cell susceptibility to pharmacological compound, in addition to significantly affect cell exposition to intracellular oxidative stress, demonstrating novel biological function(s) for these spliced proteins.

MATERIAL AND METHODS

Depletion of *i2s* by exon 5b shRNA in human cells and cellular metabolism

Primers for shRNAmir specific to exon 5b of UGT1A (**Supplemental Table 1**), were designed using RNAi Central (http://cancan.cshl.edu/RNAi_central/). Additional details are provided in **Supplemental Experimental Procedures**. HEK293T were transfected with pGIPZ (shRNAmir non-target control or targeting exon 5b) and Trans-Lentiviral Packaging Mix using Arrest-In from the GIPZ system (Open Biosystem, Ottawa, ON). A multiplicity of infection of 1 was employed to infect HT115 cells (European Collection of Cell Cultures). Isoform-specific quantitative PCR strategies were used to confirm v2/v3 mRNA depletion (Bellemare et al., 2010c). Enzymatic metabolism assays for SN-38 glucuronidation were performed using 50 µg of cell homogenates incubated for 60 min at 37°C with 200 µM of SN-38 and rates of SN-38 glucuronide formation were measured by MS.(Gagne et al., 2002)

Cell viability assay and intracellular ROS detection

HT115 cells were treated with increasing concentrations of SN-38 and AlamarBlue (Life technologies, Burlington, ON) was added to assess cell viability. Fluorescence was measured (565 nm/ 585 nm). IC₅₀ values were calculated using Prism 5 (GraphPad Software). To assess ROS levels, HT115 cells were treated with SN-38 for 48 h and incubated with 5 µM CellROX Deep Red Reagent (Life Technologies, Burlington, ON) (30 min at 37°C). The fluorescence of labeled cells was measured by flow cytometry (BD FACSCanto II, BD Biosciences, San Jose, CA).

Global gene expression analysis

Whole-genome gene expression was assessed with total RNA from HT115 cells (KD vs. reference). Total RNA was amplified, labeled, and hybridized to HumanHT-12 v4 Expression

BeadChips (Illumina, San Diego, CA) at the Genome Quebec Innovation Centre from McGill University. Data were analyzed using FlexArray 1.6.1 software, and the Lumi algorithm was used to preprocess microarray data by variance-stabilized transformation and quantile normalization and utilized the algorithm Significance Analysis of Microarrays.(Du et al., 2008) Differentially expressed genes were then subjected to functional clustering analysis using the Ingenuity Pathway Analysis system. Microarray data were submitted to GEO (accession number GSE48118 at <http://www.ncbi.nlm.nih.gov/geo/>).

Global proteomic analysis using SILAC-MS

Detailed experimental of stable isotope labeling of amino acids in cell culture (SILAC) is provided in **Supplemental Experimental Procedures**. HT115 were double SILAC labeled using stable isotope of lysine and arginine ($^{13}\text{C}_6$ Lys and $^{13}\text{C}_6$ $^{15}\text{N}_4$ Arg; Cambridge Isotope Laboratories, St-Leonard, QC). Equal amounts of light (non-target) and heavy (knockdown) cells were mixed, washed and lysed (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.3 % sodium deoxycholate, 1 % (w/v) Igepal, 1 mM EDTA, complete protease inhibitor). Trypsin digested peptides were analyzed using isoelectric focusing as described (Gagne et al., 2012) and identified using a TripleTOF 5600 (ABSciex, Concord, ON). Proteins were identified/quantified using Protein Pilot v4 software (Paragon and Progroup algorithms; Shilov, ABSciex) and search using MASCOT (Matrix Science) in the human protein database (Uniref May 2012). Data were submitted to PRIDE (accession number PXD000295).

Global analysis of protein-protein interactions

Immunoprecipitation of UGT1A isoforms and protein complexes were carried out on S9 fractions of human kidney or intestine (Xenotech, Lenexa, KS) and HT115 cell lysates. IP was performed on 1 mg of total protein lysate (**Supplemental Experimental Procedures**) with 4 μg

of antibody (anti-UGT1A_i1 #9348 or _i2 #4863) and with protein G-coated magnetic beads (Dynabeads, Life Technologies, Burlington, ON) for 15 h at 4°C. Protein identification was established using a TripleTOF 5600 after on-beads trypsin digestion. The observed endogenous interactions were confirmed by co-IP experiments with HT115 lysates. Protein interaction complexes were fixed using 0.125% formaldehyde. Cells were lysed (150 mM NaCl, 1 % (w/v) Igepal, 1 mM DTT, 175 mM Tris-HCl pH 7.4, complete protease inhibitor) and IP was performed on supernatants using 200 µl of beads (mix with 8 µg of anti-UGT1A_i1 or i2). Protein complexes were eluted and subjected to SDS-PAGE, and the presence of interacting partners was revealed on a nitrocellulose membrane with a specific antibody.

Statistical analysis

All analyses were performed in triplicate, and data were derived from at least three independent experiments unless otherwise specified. Results are expressed as the mean ± S.D. Statistical analysis was performed with 2-tailed Student's t-tests with $P < 0.05$ considered statistically significant. Statistical analysis for microarrays was performed with the algorithm Significance Analysis of Microarrays, and $P < 0.01$ was considered statistically significant.

RESULTS

Depletion of endogenous i2 splice forms modifies cell metabolism and drug response

We developed a stable KD cellular model based on an i2-specific shRNAmir cloned into a lentiviral vector designed to deplete endogenous v2/v3 transcripts encoding i2 proteins in the HT115 colon cancer cell model. Eight shRNAmirs were tested (**Supplemental Table 1 and Figure 1A**) and the clone that provided the best KD was used. The shRNA p27 (start position at nucleotide 27 of exon 5b) led to a 49 % decrease ($P=0.005$) in v2/v3 mRNA levels without significantly affecting v1 mRNA levels (**Figure 1B**). Depletion of i2 was thus specific and stable (from 20 to 200 days post-infection). Compared with non-target shRNA-infected HT115 cells (corresponding to the reference cell line), drug glucuronidation activity increased 126 % ($p=0.008$) for i2-depleted cells (HT115 KD) (**Figure 1B**). This increase in drug inactivation correlated with enhanced viability of cells, i.e., the 50 % inhibition concentration value (IC_{50}) of SN-38 for HT115 KD cells ($0.45 \pm 0.05 \mu\text{M}$) was higher than that for the reference ($0.22 \pm 0.03 \mu\text{M}$; $P=0.006$) (**Figure 1C**). This increased viability induced by i2 KD was particularly significant between $0.08 \mu\text{M}$ and $0.8 \mu\text{M}$ of SN-38, which correspond to doses required to achieve a 50 % inhibition in cell growth.

Global gene expression profiling of functional KD of UGT1A_i2 and altered production of reactive oxygen species (ROS)

Compared to the reference cells, microarray gene expression profiling of HT115 KD cells revealed significant modulation of 207 genes and of 586 genes (fold change ≥ 1.2 , $P < 0.01$), under standard culture conditions and upon treatment with SN-38 ($0.5 \mu\text{M}$, 48 h), respectively. **Figure 2** shows heatmap data for the 50 most differentially expressed genes in cells treated or not with SN-38. Among these genes, hemoglobin alpha genes (*HBA1/HBA2*) were upregulated

significantly ($P<0.01$) (**Supplemental Table 2**). *HBA1/HBA2* expression was confirmed in peripheral blood cells from few healthy donors and we also demonstrated their expression in tissues from the gastrointestinal tract including sections of the intestine, healthy colon tissue and colon tumors as well as in HT115 (**Figure 3A**). Compared with the reference cell line, HT115 KD cells further displayed upregulated expression of HBA2 under basal condition (1.3 fold, $P=0.05$) and of HBA1 after drug exposure (5.5 fold; $P=0.01$), which was subsequently confirmed at the protein level (**Figure 3**). Because the function of HBA proteins has been linked to cellular antioxidant potential (Widmer et al., 2010), we evaluated if depletion of *i2s* influences cellular oxidative stress response by quantification of intracellular ROS. HT115 KD cells had greater antioxidant potential compared with the reference cell line, as ROS formation was reduced by >30 % ($P<0.05$) following SN-38 treatment (**Figure 3C**), consistent with previous reports indicating that SN-38 induces cellular oxidative stress (Conklin, 2004; Timur et al., 2005).

Global quantitative protein expression profiling associated with functional KD of *i2*

We used the unbiased SILAC-MS approach to quantify altered protein expression upon *i2* depletion (**Figure 4A**). We first used lysates of cells from SILAC to demonstrate essentially full incorporation (98.3%) of targeted isotopic labels. We next conducted a global MS study to quantitatively compare the proteomes of SILAC-labeled HT115 KD cells and unlabeled HT115 cells grown without drug treatment. We detected a significant increased expression for 11 proteins (>1.2 fold; $P<0.05$) (**Figure 4B**). We then applied a reliable bioinformatics approach to establish potential functional network based on the regulated proteins (**Supplemental Figure 1**) and reveal that almost all proteins we identified are related to the intrinsic antioxidant defense network, including superoxide dismutase 1 (SOD1) and others (**Table 1**). **Supplemental Figure**

2 presents a representative MS spectrum of SOD1 peptides. Western blotting confirmed that SOD1 levels increased in HT115 KD cells (**Figure 4C**).

Interaction proteomics experiments reveal antioxidant enzymes catalase and peroxiredoxin 1 as specific partners of i2 isoforms in human tissues and HT115 cells

Because i1 enzymes and i2 proteins function as oligomers, we tested the hypothesis that i2s interact with proteins other than i1s and specific partners not interacting with i1 enzymes, some of which may participate in oxidative stress pathways. We used IP-coupled to MS using specific antibodies for each class of UGT1A isoforms to depict i1- and i2-associated interactomes (**Figure 5A**), and observations were based on three independent IP-MS/MS experiments for each antibody. As specific partners of i2, notably in the intestine, we identified for the first time the enzyme catalase, which has the highest turnover number of all antioxidant enzymes (Valko et al., 2006), as well as the antioxidant enzyme peroxiredoxin 1 (Prdx1), which reduces hydrogen peroxide and alkyl hydroperoxides (Imlay, 2008). In i2 immunoprecipitates from the intestine, we detected several unique peptides specific to catalase and Prdx1 that were not identified in anti-i1 immunoprecipitates (**Supplemental Table 3**). Furthermore, co-IP using specific antibodies directed against catalase and Prdx1 confirmed their association with i2s (but not i1s) (**Figure 5B**).

DISCUSSION

UGTs are major mediators in conjugative metabolism, and variations of this pathway have been clearly shown to influence drug response and treatment outcomes (Lazarus et al., 2009; Guillemette et al., 2010). In keeping with this view, we sought to assess if modulation of endogenous levels of regulatory i2 proteins, through RNA interference-mediated repression of i2 spliced isoforms, would impact cellular metabolism and pharmacological response to an anticancer agent (SN-38) primarily metabolized by UGT1A enzymes. A partial depletion of i2 isoforms was achieved and sufficient to significantly enhance glucuronidation of the drug. Furthermore, i2-depleted cells were also more viable than the reference cell line upon drug treatment and displayed increased resistance to this anticancer agent that cannot be attributed to a direct increase of active i1s. As we previously showed that i2s act to repress glucuronidation activity through protein-protein interactions with i1s, (Bellemare et al., 2010b) this partial depletion in i2 splice forms was expected to lead to increased availability of the active i1 enzymes, with a consequent overall increase in cellular UGT1A-mediated glucuronidation and a meaningful impact on the pharmacological response to a drug, e.g., an improvement in cell viability. Our data are clearly supportive of a negative regulatory role for i2s upon drug glucuronidation and have the potential to affect the pharmacological response to drug and likely contribute to the observed interindividual variability in drug metabolism and response.

Our work also provides the first evidence that i2s participate in the regulation of oxygen and ROS metabolic processes through direct protein-protein interactions with antioxidant enzymes and by affecting their expression at the mRNA and protein levels. Microarrays revealed upregulated expression of hemoglobin alpha *HBA1* and *HBA2* upon i2 depletion. *HBA* products are well known for their oxygen transporter function, but they also have an antioxidant function

as they bind cellular ROS (Reeder, 2010; Widmer et al., 2010). These genes are primarily expressed in peripheral mononuclear blood cells (Haas et al., 2011) and, as demonstrated in our present work, are also expressed throughout the gastrointestinal tract with relatively higher expression in duodenum and jejunum, consistent with their role as antioxidant enzymes. Our data also demonstrate their expression in colon tumors and in the related HT115 cellular model. The increased expression of HBA in i2-depleted cells was further associated with a significant reduction in intracellular ROS formation, thus supporting a potential link between i2s and the oxidative stress pathway. Accumulation of ROS is a vital signal that initiates diverse cellular processes, and it is also plausible that altered ROS homeostasis might provide a critical signal for inducing antioxidant pathways such as HBA. Likewise, a change in intracellular ROS levels similar to the one observed upon i2 depletion was previously shown to influence cellular autophagy in U2OS osteosarcoma cells (Bensaad et al., 2009). In addition, data from SILAC-MS also pointed to an enhanced expression of several other proteins that have network functions associated with antioxidant properties. This is in line with an interrelation among factors of the cellular defense system that is not only comprised of phase I and phase II detoxification enzymes such as UGTs but of many of the proteins shown to be affected by i2 depletion, including SOD1 and others (**Table 1**). Defining the exact mechanisms by which these changes occur requires further experiments.

By applying an unbiased MS-based IP proteomics analysis to HT115 cells and human tissues, our experiments reveal for the first time a direct protein-protein interaction between i2s and antioxidant enzymes such as catalase and Prdx1, further confirmed by co-IPs. Whereas most protein complexes are usually isolated through overexpression of a tagged exogenous protein within cells, the strength of our approach relies on targeting endogenous proteins of human

intestine and kidney where i2 expression was previously demonstrated (Levesque et al., 2007; Bellemare et al., 2011). In addition, we show an i2-specific interaction with catalase and Prdx1, especially in the intestine. Both the catalase and Prdx1 pathways are involved in the elimination and modulation of local H₂O₂ levels that play a pivotal signaling role in several cellular processes. This previously unknown capacity of i2 proteins to interact with antioxidant proteins evokes their potential to regulate the activities of these enzymes. Both catalase and Prdx1 must form a tetramer to achieve activation, and thus it is possible that binding of i2s to these enzymes may serve as a way of inhibiting their activities by perturbing their oligomerization. Clearly such a property of i2s is biologically relevant, as we have shown that a portion of i2s is cytoplasmic, in contrast to i1s that are resident of the ER (Levesque et al., 2007; Bellemare et al., 2011). The cytoplasmic distribution of Prdx1, catalase and i2 isoforms is therefore consistent with i2s interfering with their oligomerization and the biological functions of these ROS scavengers (Leon et al., 2006; Rho et al., 2008).

We conclude that, in addition to regulating drug metabolism and response (Figure 6), the endogenous levels of i2 splice isoforms may contribute to controlling oxidative stress and consequently numerous signaling pathways, thereby affecting several cellular phenotypes.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: M. Rouleau, J. Bellemare and C. Guillemette

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Performed data analysis, supervised the project: M. Rouleau, J. Roberge and C. Guillemette

Wrote the manuscript: M. Rouleau, J. Roberge, C. Guillemette

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FOOTNOTES

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The authors have no conflict of interest to disclose.

FIGURE LEGENDS**Figure 1. Effect of i2 depletion on pharmacological response to the anticancer agent SN-38.**

A) shRNAmir targeting exon 5b or its 3' untranslated region. Eight shRNAmir were tested and the most efficient is represented with a solid black line. **B) Partial depletion of endogenous i2 enhances cellular glucuronidation activity.** Repression of transcripts v2/v3 (left axis) is confirmed by qPCR and normalized to RPLP0 mRNA levels with no changes in v1. Glucuronidation activity (right axis) is displayed. **C) Increased cell viability upon i2 KD.** Fluorescence values were converted into percentage of viable cells (using AlamarBlue) compared with non-treated cells and GraphPad Prism 5 was used to calculate IC₅₀ values. Data represent the mean ± S.D. of at least three independent experiments, with each sample in triplicate or quadruplicate. **P*<0.05; ***P*<0.01; ****P*<0.001.

Figure 2. Gene expression analysis upon i2 depletion.

Heatmap displaying the top 50 most differentially expressed genes (fold change > 1.2, *P*≤0.01) after i2s depletion in HT115 cells either as **A) Genes modulated by i2 KD under the standard culture condition** or **B) Genes modulated by i2 KD upon pharmacological treatment (SN-38 0.5 μM for 48 h)**. We used the HumanHT-12 v4 Expression BeadChips (Illumina, San Diego, CA). Clustering analysis was performed with Cluster 3.0 and visualized using TreeView 1.1.6r2. Upregulated genes are shown in shades of red, and downregulated genes are shown in shades of green. Experiments were performed on two biological replicates.

Figure 3. Expression analysis of hemoglobin (HBA) pathways and ROS formation.

A) Quantitative PCR analysis of HBA genes expression. mRNA expression experiments were carried out with peripheral blood from healthy volunteers as a positive control of HBA

expression (n=3; left panel), in several human tissues of the GI tract (n=1 individual per tissue), colon tumors (n=11; middle panel) and in untreated and treated HT115 cells (SN-38 0.5 μ M, 48 h) (right panel). Data represent the mean \pm S.D. normalized to RPLP0 and GAPDH mRNA levels. **B) HBA protein expression.** Western blotting carried out with untreated cell homogenates (50 μ g) revealed increased expression of HBA (anti-HBA 1:200, Santa Cruz, Santa Cruz, CA) in HT115 i2-depleted cells. Calnexin was used as the control protein (anti-calnexin 1:2000, Stressgen, Burlington, ON). **C) Intracellular levels of reactive oxygen species (ROS).** ROS formation was measured using Cell ROX deep red fluorescent reagent (Life Technologies, Burlington, ON) and flow cytometry. Experiments were performed three times, with each sample in triplicate. * $P \leq 0.05$; ** $P \leq 0.01$.

Figure 4. SILAC-MS reveals differentially expressed proteins in i2-depleted HT115 cells.

A) Schematic overview of the experimental design. Non-target shRNA cells were grown in light medium (lysine and arginine, ^{12}C ^{14}N) whereas i2-depleted cells were grown in heavy medium (lysine and arginine, ^{13}C ^{15}N). Equal amounts of light and heavy cells were mixed, lysed, digested with trypsin for subsequent isoelectric focusing on an immobilized pH gradient, fractionated in 14 sections, and peptides identified by MS. Proteins were identified with high confidence using Scaffold software (minimum protein identity 95 %; at least two unique peptides identified; peptide probability >95 %). Peptides were quantified by directly comparing relative intensities of the light and heavy forms of each peptide identified. **B) Differentially expressed proteins in i2-depleted HT115 cells.** Ratios are expressed as protein enrichment in heavy culture (HT115 KD) relative to light culture (reference) (H:L). **C) Confirmation by western blotting of SOD overexpression in untreated cell homogenate.** 50 μ g were used with the anti-

SOD1 (1:2500, Stressgen, Burlington, ON) and the control (anti-calnexin 1:2000, Stressgen, Burlington, ON). hSOD1: human SOD1; mSOD1: mouse SOD1.

Figure 5. UGT1A isoform-specific interactomes. A) Flow diagram of immunoprecipitation (IP) of protein complexes coupled to mass spectrometry (MS). Human tissues (kidney and intestine from Xenotech, Lenexa, KS) or HT115 cells (1 mg) were mixed with anti-UGT1A_i1 or _i2 to specifically immunoprecipitate protein complexes. In-solution trypsin digestion of protein complexes and peptides identification was performed with a TripleTOF 5600 MS using the same criteria described for the SILAC experiment. Experiments were performed in triplicate for each tissue or cell line and each antibody. **B) Co-IP of endogenous proteins confirms catalase and Prdx1 that associate specifically with i2s.** Protein complexes were fixed using 0.125 % formaldehyde, immunoprecipitated using an isoform-specific antibody (8 µg of anti-i1 (#9348) or -i2 (#4863) or negative control (8 µg rabbit immunoglobulin)). Western blotting was used to detect the interacting partners using antibodies directed against catalase (1:1000; Genscript, Piscataway, NJ) and Prdx1 (1:5000; Upstate, Billerica, MA).

Figure 6. Dual roles for i2 isoforms in the cellular defense system. A) Modulation of drug response. UGT1A_i2s act as negative regulators of glucuronidation activity by directly interacting with i1-conjugating enzymes, thereby reducing drug inactivation that led to increased cell viability and decreased intracellular ROS levels, thereby directly affecting cell responses to drug. **B) Role in oxidative stress pathways.** The expression of antioxidant enzymes such as SOD1 and others is affected by i2 depletion. Furthermore, i2s influence antioxidant enzymes such as catalase and Prdx1, through direct protein-protein interaction that could impair their oligomerized active state and likely affecting cell exposure to ROS.

Table 1. SILAC-MS experiments reveal 11 proteins differentially expressed upon knockdown of endogenous i2.

Gene symbol	Description	Molecular function	Relation to oxidative stress	# of unique peptides	SILAC ratio (H:L) ^a	p-value	Reference
WDR1	WD repeat-containing protein 1	Actin binding	Interaction with KEAP1	17	1.21	0.041	(Hast et al., 2013)
AGR2	Anterior gradient protein 2 homolog	Dystroglycan binding	Member of thioredoxin superfamily	8	1.22	0.035	(Lepreux et al., 2011)
MYOF	Myoferlin	Phospholipid binding		10	1.22	0.036	
SELENBP1	Selenium-binding protein 1	Selenium binding	Modulation of cell H ₂ O ₂ sensitivity	23	1.23	<0.001	(Pohl et al., 2009)
EPHA2	Ephrin type-A receptor 2	Kinase	Modulation of intracellular H ₂ O ₂	5	1.24	0.048	(Buricchi et al., 2007)
CCT3	T-complex protein 1 subunit gamma	Molecular chaperone/Foldase	Folding machinery for cytosolic proteins	26	1.25	0.019	
ACY1	Aminoacylase-1	Hydrolase	Tumor suppressor role in iron-induced rat renal cell carcinoma	9	1.25	0.028	(Zhong et al., 2009)
ETHE1	Ethylmalonic encephalopathy protein 1, mitochondrial	Hydrolase	Sulfur dioxygenase activity	11	1.26	0.003	(Tiranti et al., 2009)
GART	Trifunctional purine biosynthetic protein adenosine-3	Ligase	Purine metabolism	17	1.27	0.007	
ALDH2	Aldehyde dehydrogenase, mitochondrial	Oxidoreductase	Oxydation of acetaldehyde	12	1.32	0.012	(Ohta et al., 2004)
SOD1	Superoxide dismutase [Cu-Zn]	Oxidoreductase	Removal of superoxide radicals	5	1.39	0.020	(Jackson, 2006)

^aH:L : ratio of heavy and light labeled amino acids

Figure 1

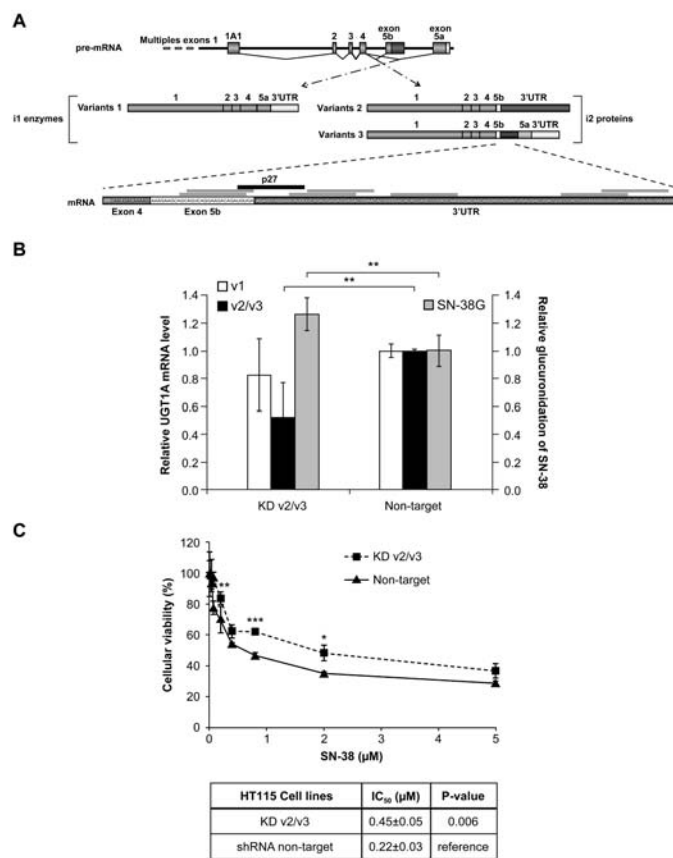


Figure 2

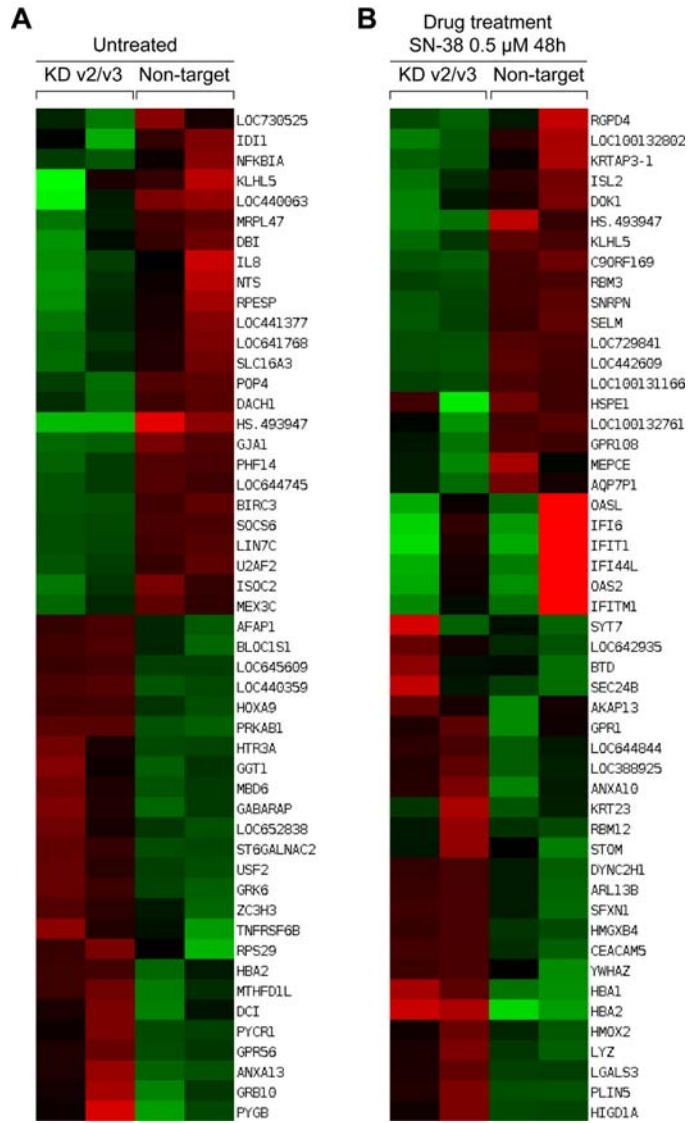


Figure 3

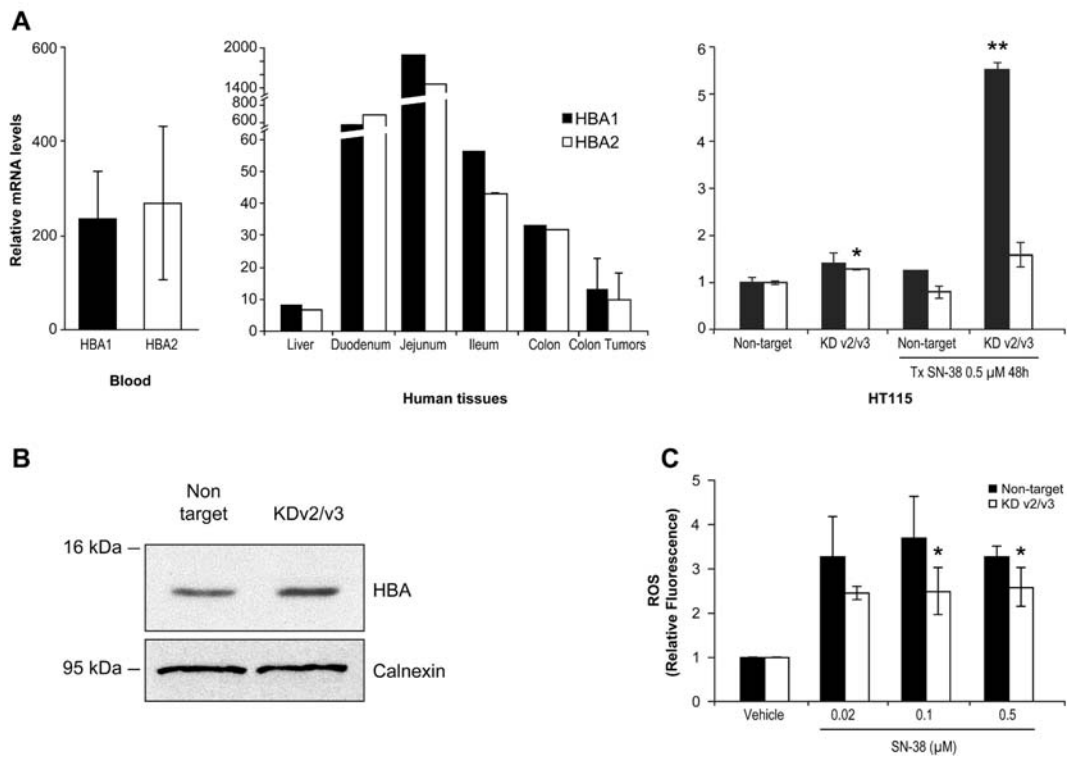


Figure 4

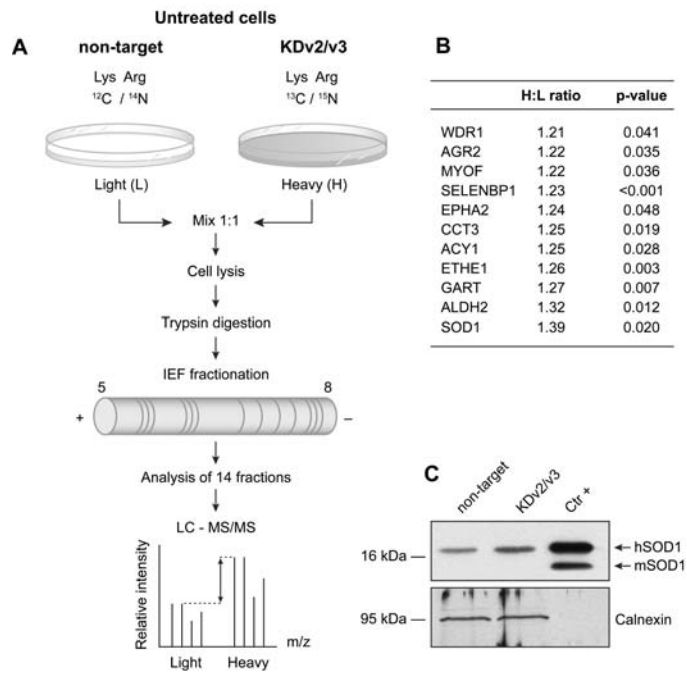


Figure 5

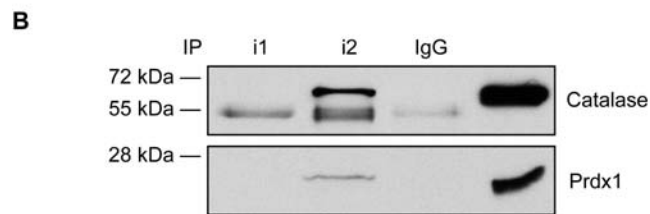
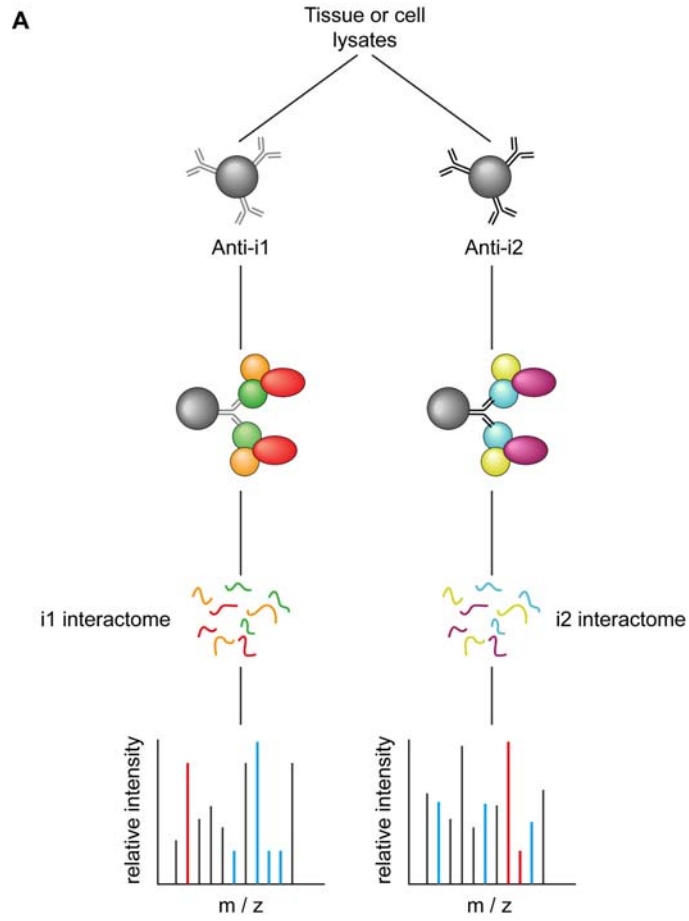
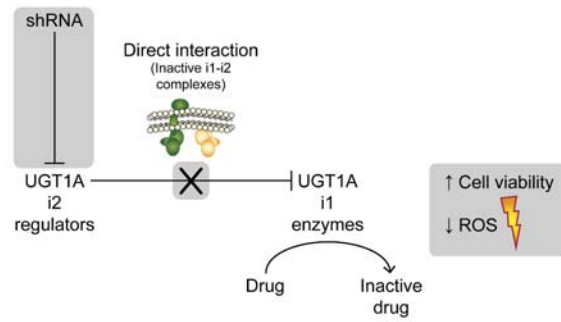


Figure 6

A) Drug response



B) Oxidative stress

