**Results:**

GFPT2 expression level was associated with cell stress and it could react to the cell reduced glutathione (GSH) level.

XXX et al. reported that overexpression of GFPT2 enhances cell survival following H2O2 treatment [REF]. We treated MDAMB231 cells with H2O2 and observed a significant increase of GFPT2 RNA expression **(Fig.Glutathione A)**. We also noted, at the time point where GFPT2 was upregulated, total glutathione in cells was not changed **(Fig.Glutathione B)** while GSH was significantly decreased **(Fig.Glutathione C)**. The D492 cells possessed higher amount of GSH than D492M and D492HER2 **(Fig.Glutathione D)** [REF] which indicated the cell stress was potentially higher in D492M and D492HER2 than D492. After a gene-metabolite correlation analysis of the NCI60 cancer cell line panel, we noticed a significant negative correlation between GFPT2 and GSH **(Fig.Glutathione E)**. We hypothesized that GFPT2 expression is associated with, and likely reacts to, cell stress. This was confirmed by the significant decrease of GFPT2 RNA expression after treating MDAMB231 cells with GSH **(Fig.Glutathione F)**. These results indicated GFPT2 can sense and react to the GSH level which is an indicator for cell stress.

Knockdown of GFPT2 broke the balance of REDOX homeostasis and triggered ways other than *de novel* glutathione synthesis to compensate for the loss of GSH. SQOR, possibly amongst others, was involved in this process.

GFPT2 catalyzes glutamine to glutamate which is used for glutathione *de novel* synthesis. GFPT2 expression has previously been associated with increased glutamate in the NCI60 cancer cell line panel [REF]. A drop in intracellular glutamate, the second product of GFPT2, was also observed for D492 and D492M following knockdown of GFPT2, although this was not significant in D492HER2 [REF] **(Figure X)**. We hypothesized that GFPT2 might influence the cellular redox state by modulating intracellular glutathione via its precursor glutamate. To test this, we knocked down GFPT2 via siRNAs and observed an increase on both GSH/GSSG ratio and total glutathione level, however, only one siRNA passed the significant threshold among all three cell lines **(Supplementary Fig. A-F)**. This result was contradictory to the hypothesis where GFPT2 and glutathione should have a positively causal relationship. We hypothesized the unexpected increase of glutathione after GFPT2 knockdown was caused by genes involved in glutathione homeostasis and recycling. We noticed particularly NFE2L2, NQO1 and SQOR were highly expressed in D492 than in D492M and D492HER2 **(Supplementary Fig. G-J)**. These genes might be at least partially responsible for the lower cell stress in D492 compared to D492M and D492HER2 and GFPT2 knockdown might affect these genes. We tested the expression level of these three genes after GFPT2 knockdown and NFE2L2 showed inconsistent trends among the three cell lines **(Supplementary Fig. K-M)** while NQO1 showed a decreasing trend but did not pass the significant threshold for D492HER2 **(Supplementary Fig. N-P)**. Only SQOR showed consistent decreases in all four cell types and the decrease of SQOR was confirmed with two siRNAs **(Fig.Glutathione G-N)**. SQOR catalyzes the oxidation of hydrogen sulfide (H2S) and is believed to use glutathione as electron acceptor *in vivo*. SQOR is a glutathione-sensitive gene. By altering its expression, SQOR facilitates the adjustment of glutathione level inside cells [REF]. All in all, these data suggested GFPT2 regulated glutathione homeostasis not only by regulating the glutamate level, but also by affecting SQOR gene expression.

**Discussion points:**

After H2O2 treatment, total glutathione (*de novel* synthesis) was not changed while GSH/GSSG ratio was reduced significantly.

After H2O2 treatment, catalase and glutathione peroxidase and perhaps changes to their gene expression.

Glutathione *de novo* synthesis would be expected to be compromised with GFPT2 knockdown, because there was a clear slowdown into the HBP pathway in the previous labelling experiments with GFPT2 gene suppression and this was supported with lower glutamate levels.

The increase on GSH/GSSG ratio after GFPT2 knockdown indicated cells were under stress and a break of the REDOX homeostasis after knockdown of GFPT2.

Due to limited time, we could not overexpress GFPT2 in cells to further address the role of GFPT2 in regulating REDOX homeostasis.

Further investigation is needed to understand the mechanism of GFPT2 regulating SQOR.

1-you impact H2S levels that influence GSH/GSSG ratio. This would however not lead to enhanced GSH de novo synthesis- only increase GSH.

2-you influence ubiquinol regeneration and thus flow through the electron chain and oxygen consumption. This results in glutamate shunting into increased proline and glutathione synthesis. (This we have seen before in other projects)

1-slowdown of hexosamine biosynthesis enhances flux through the PPP that increases GSH.

-measure NADPH

-stable isotope labelling experiment.

2- reduced flow through hexoseamine biosynthesis reduces sulfate demand. This effects SQRDL and increases H2S that effects GSH and reduces ubiquinol with a slowdown of the ETC.

-check proline, cysteine and homocysteine, methionine, SAM and GSH GSSG levels in cells in LCMS data. Probably would have to check in positive mode if you have that.

-measure sulfate.

Studies have shown that GFPT2 is multifunctional in cells. It catalyzes the formation of UDP-GlcNAc to regulate O-GlcNAcylation in cells [REF]; it has also been suggested to influence glucose uptake [REF] and Reactive Oxygen Species (ROS) sensitivity of cells [REF]. ROS in cancer has been widely studied and holds the potential to be a critical anticancer therapy.

*Figure legend below*

Diagram

Description automatically generated

***Figure X.*** GFPT2 was associated with cell stress. **(A)** GFPT2 RNA expression was significantly upregulated after H2O2 treatment in MDAMB231. **(B)** The total glutathione level did not change after H2O2 treatment in MDAMB231. **(C)** The GSH level significantly decreased after H2O2 treatment in MDAMB231. **(D)** GSH level was significantly higher in D492 than in D492M and D492HER2. **(E)** Gene-metabolite correlation analysis of the NCI60 cancer cell line panel indicated a negative correlation between GFPT2 and GSH. **(F)** Treatment with GSH significantly downregulated the GFPT2 gene expression in MDAMB231. **(G-J)** SQOR RNA expression was significantly downregulated in D492 (G), D492M (H), D492HER2 (I) and MDAMB231 (J) after knocking down of GPFT2 with the first siRNA. **(K-N)** SQOR RNA expression was significantly downregulated in D492 (K), D492M (L), D492HER2 (M) and MDAMB231 (N) after knocking down of GPFT2 with the second siRNA. **\***: p < 0.05; **\*\***: p < 0.01; **\*\*\***: p < 0.001.

**Data used:**

**A:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\21 H2O2-Treatment-GFPT2-GFPT1-NFkB\RTqPCR\_MDAMB231\_H2O2\_Starvation\_GFPT2\_GFPT1\_ACNB-GAPDH\_20.10.2020.xlsx*

**B&C:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\16 GSH.GSSG.Ratios\GSH\_GSSG\_Ratios\_MDAMB231\_Summary\_2020.12.02-H2O2.xlsx*

**D:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Glutathione\Panel4\ RawData-Acidic-wt1-GSH.xlsx (C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\Glycan precursor analysis\KDexperimentRepeat\_16.07.2019\QIONG\AcidicNeg\_Knockdown\_Metabolomics\_3Scr\_template.xlsx)*

**E:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\NCI60\Rfiles\ GFPT1\_GFPT2\_metabolite\_correlation\_AUGUST2020-QIONG.R*

**F:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\25 MDA-MB-231\GFs-depleting\ RTqPCR\_MDAMB231\_GFs\_20.11.2020-Glutathione-GFPT2.xlsx*

**J&N:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\25 MDA-MB-231\siGFPT2-FBS\ RTqPCR\_MDAMB231\_siGFPT2\_RNA1\_08.09.2020-GFPT2.xlsx & RTqPCR\_MDAMB231\_siGFPT2\_RNA2\_12.09.2020-GFPT2.xlsx*

**Figure Location:**

**A:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\21 H2O2-Treatment-GFPT2-GFPT1-NFkB\Figures*

**B&C:** C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Glutathione\Panel2-3-H2O2-Treatment-Glutathione\ Glutathione-level-H2O2-treatment-MDAMB231.xlsx

**D:** C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\Glycan precursor analysis\KDexperimentRepeat\_16.07.2019\QIONG\Figures\Glutathione\WT\ 2020-02-06 11-30-54 Glutathione.Red Glutamic.Acid.IS wt1 bar.plot.tiff

**E:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\NCI60\Figures*

**F:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Glutathione\Panel6\ GFPT2-level-Glutathione-treatment.xlsx*

**J&N:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\25 MDA-MB-231\siGFPT2-FBS\Figures*

**Supplementary Figure X**

Diagram

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***Supplementary Figure X.*** Increase of GSH/GSSG ratio and total glutathione level after knockdown of GFPT2. **(A-C)** GSH/GSSG ratios after knockdown of GFPT2 with two siRNAs in D492 (A), D492M (B) and D492HER2 (C). **(D-F)** Total glutathione levels after knockdown of GFPT2 with two siRNAs in D492 (D), D492M (E) and D492HER2 (F). **(G-H)** SQOR was highly expressed in D492 at both RNA (G) and protein levels (H). The protein expression of SQOR was from LFQ proteomic analysis. SQOR was not detected in the SILAC proteomic experiment. **(I)** NFE2L2 RNA expression was higher in D492 than in D492M and D492HER2 based on RT-qPCR. NFE2L2 was not detected in both LFQ and SILAC analysis. **(J)** NQO1 protein level was higher in D492 according to both LFQ and SILAC detections. **(K-M)** NFE2L2 RNA expression in D492 (K), D492M (L) and D492HER2 (M) after GPFT2 knockdown. **(N-P)** NQO1 RNA expression in D492 (N), D492M (O) and D492HER2 (P) after GPFT2 knockdown. **\***: p < 0.05; **\*\***: p < 0.01; **\*\*\***: p < 0.001.

**Data used:**

**A&D:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\16 GSH.GSSG.Ratios\ GSH\_GSSG\_Ratios\_EMH\_Summary\_2020.03.30 – NewCal.xlsx*

**B&E:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\16 GSH.GSSG.Ratios\ GSH\_GSSG\_Ratios\_EMH\_Summary\_2020.04.21 - NewCal - D492M.xlsx*

**C&F:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\16 GSH.GSSG.Ratios\ GSH\_GSSG\_Ratios\_D492HER2\_Summary\_2020.09.13-repeat.xlsx*

**Figure Location:**

**A&D (B&E, C&F):** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Glutathione\SupplementaryFigure.GSH-GSSG-Ratios\ RawData-EMH-GSH-GSSG-Ratios.xlsx*

MDAMB231:

*C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Glutathione\SupplementaryFigure.GSH-GSSG-Ratios\***RawData-EMH-GSH-GSSG-Ratios.xlsx**