**Results:**

In literature, the regulation of GFPT2 has been associated with several growth factors and transcriptional factors including EGF, TGFB, TNF, NFkB, SIRT6, sXBP1 and KRAS etc. [REF]. GFPT2 is not only regulated by many regulators but also regulating signaling regulators via glycosylation [REF]. These indicated GFPT2 signaling regulation is due to signaling cross talks and is more complicated than just limited pathways involved. In order to identify the type of signaling deregulation associated with GFPT2 expression，we collected cultured medium for D492HER2 and D492 and analyzed the secretome with respect to alternate signaling pathways. We found common changes in altered abundance of secreted proteins in D492 and D492HER2 involved in TGFB, IGF, TNF and EGF signaling etc. **(Figure Signaling A)**. TGFB, TNF and EGF signaling have ready been confirmed in literature to regulate GFPT2 [REF]. Besides, many earlier studies regarding GFPT2 have been focusing on the role of GFPT2 in Diabetes [REF]. Hence, we decided to focus on the IGF signaling. RNA expression of GFPT2 decreased after insulin removal in MDAMB231 **(Figure Signaling B)**. The protein expression of IGF1R is higher in D492HER2 than D492 **(Figure Signaling C)**. To take into account regulation of these pathways, with focus on IGF signaling, we performed phosphoproteomic analysis of D492HER2 and D492 and found activation of, amongst others, ERK/MAPK signaling **(Figure Signaling D)** and kinase enrichment of MAPKAPK2, GSK3B, and PKCα etc. **(Figure Signaling E)**. GSK3B is a downstream regulator of ERK/MAPK signaling and inhibited by its activation [REF]. The expression of GSK3B is higher in D492 at both RNA and protein levels **(Figure Signaling F-G)**. GSK3B knockdown resulted in increased GFPT2 expression at RNA level **(Figure Signaling H-K)**. GSK3B regulates NFkB (p65, RELA) that has previously been shown to modulate GFPT2 [REF]. However, siRNA knockdowns of NFkB (p65, RELA) did not suppress GFPT2 **(Supplementary Figure X A-B)**. PKCα is a common EMT marker and highly expressed in D492HER2 and D492M than in D492 **(Figure 1A)**. It was one of the enriched kinases and associated with ERK/MAPK signaling [REF]. We decided to check if it was playing any roles in GFPT2 regulation by knocking down PKCα via siRNA and surprisingly noticed an increase of GFPT2which was confirmed in two cell lines **(Supplementary Figure X C-D)**.

**Discussion points:**

Lots of signaling pathways are involved in the regulation of GFPT2.

Knockdown of RELA and PKCα makes it more convincing that the regulation of GFPT2 is very complicated.

The importance of RELA to GFPT2 might depend on the cell system.

PKCα affects GFPT2 independent of ERK/MAPK signaling.

Interfering PKCα affects oxidative stress, which might be responsible for the increase of GPFT2.

*Figure Legend below.*

Chart, box and whisker chart

Description automatically generated

***Figure X.*** Signaling regulation of GFPT2. **(A)** Secretome of D492HER2 and D492 revealed a list of growth factors that were secreted differently between these two cell lines (FDR < 0.05, Fold change > =2). **(B)** Insulin removal decreased GFTP2 RNA expression in MDAMB231 cell line. **(C)** The protein level of IGF1R is higher in D492HER2 than in D492 based on SILAC proteomic data. **(D)** Top 8 of the Ingenuity Canonical Pathways from the phosphoproteomic data analysis. Pathways activated in D492HER2 were in orange while pathways activated in D492 were in blue. Dots referred to the absolute value of activation Z-scores. Pathways were listed based on p value. **(E)** Motif enrichment from Perseus (Version 1.6.14.0) suggested a list of kinases behaving differently in D492HER2 compared to D492. **(F-G)** RNA (F) and protein (G) expression of GSK3B in D492HER2 vs. D492. **(H)** Knockdown efficiency for GSK3B with the first siRNA. **(I)** GFPT2 RNA expression after knockdown of GSK3B in D492 with the first siRNA. **(J)** Knockdown efficiency for GSK3B with the second siRNA. **(K)** GFPT2 RNA expression after knockdown of GSK3B in D492 with the second siRNA. **\***: p < 0.05; **\*\***: p < 0.01; **\*\*\***: p < 0.001.

**Data used:**

**A:**  *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\1 DundeeProteomicDataSet\_14112017\ProteomicDataAnalysis\_Sophie&Erika\Sophie\Secretome\_D492vsD492her2\Perseus-1.6.14.0\* *PerseusOutputData.xlsx*

**B:** *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\_30.11.2020-H14-GFPT2.xlsx*

**C:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\Panel2-3-Insulin-EGF-SILAC\ 2020-12-06 10-52-56 ExportData\_D492HER2 vs. D492.xlsx*

**D:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\11 IPA\IPA import & export\_02.12.2018\ExportFiles\PHOSPHOPROTEOMICS\HE\Data\_1\pCanonicalPathways\_HE.xls*

**E:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\2 SILIC\_DundeePhosphoproteomicsDataset\Results\Phosphoproteomics\_1\Perseus\PerseusOutputTable\3 PerseusOutPutTable\_Phosphos\_HE\_motif enrichment\_all.txt*

**F:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\25 MDA-MB-231\WT\RTqPCR\_WT\_EMH\_231\_14.10.2020\_GSK3B.xlsx*

**G:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\Panel6-7-GSK3B\2020-12-04 20-28-53 ExportData\_D492HER2 vs. D492.xlsx*

**H-K:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\18 Knockdown-siRNA1&2-GSK3B\RT-qPCR\_D492\RTqPCR\_KDefficiency\_GSK3B\_D492\_RNA1&2\_GSK3B\_GFPT2\_NFkB\_2020.05.19.xlsx & RTqPCR\_KDefficiency\_GSK3B\_D492\_RNA3\_2020.11.09-GSK3B-GFPT2.xlsx*

**Figure location:**

**A:**  *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\ Panel1-Secretome-GrowthFactors.pptx*

**B:** *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\_30.11.2020-H14-GFPT2.xlsx*

**C:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\Panel2-3-Insulin-EGF-SILAC\ 2020-12-06 10-52-56 ExportData\_D492HER2 vs. D492.xlsx*

**D:**  *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\Panel4-IPA\Bar-Plot-Dox-Plot-IPA-2020.12.05.R*

**E:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\Panel5-Perseus-MotifEnrichment\Functions\_treemap-Motif-Enrichment-Perseus-D492HER2-D492.plot.R*

**F:**  *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\Panel6-7-GSK3B\GSK3B-RNA-level-D492HER2-D492.xlsx*

**G:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\Panel6-7-GSK3B\2020-12-04 20-28-53 ExportData\_D492HER2 vs. D492.xlsx*

**H-K:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\Panel8-11-siGSK3B-GFPT2\BoxPlot\_D492\_GSK3B-GFPT2\_siGSK3B\_2020.12.05.R*

Diagram, schematic

Description automatically generated

***Supplementary Figure X.*** GFPT2 RNA expression after siRNA Knockdown of NFkB (p65, RELA) and PKCα. **(A-B)** Knockdown efficiency of NFkB (p65, RELA) (A) and GFPT2 RNA expression after knockdown of NFkB (p65, RELA) in D492HER2 (B). **(C-D)** Knockdown efficiency of PKCα (C) and GFPT2 RNA expression after knockdown of PKCα in D492HER2 (D). **(E-F)** Knockdown efficiency of PKCα (E) and GFPT2 RNA expression after knockdown of PKCα in D492M (F). **\***: p < 0.05; **\*\***: p < 0.01; **\*\*\***: p < 0.001.

**Data used:**

**A&B:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\26-KnockDown-siRNA-RELA\RTqPCR\_KDefficiency\_RELA\_D492HER2\_RNA1&2\_08.11.2020-RELA-GFPT2.xlsx*

**C&F:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\21 KnockDown-siRNA-PKCa\ RTqPCR\_KDefficiency\_PKCa\_D492HER2\_RNA1\_14.06.2020\_GFPT2\_PKCa.xlsx & RTqPCR\_KDefficiency\_PKCa\_D492M\_RNA1\_07.06.2020\_GFPT2\_UGDH\_PKCa.xlsx*

**Figure location:**

**A&B:** C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\5 ProteomicPaper\Figures&Tables in the paper\ProteomicsManuscript\_25.11.2020\Figures\Fig.Signaling\SupplementaryFigure-siRELA\siRELA\_D492HER2\_RNA1&2\_08.11.2020-RELA-GFPT2.xlsx

**C&F:** *C:\Users\lenovo\OneDrive - Háskóli Íslands\PC-HI\4 Experiments documents\_QIONG\14 Knockdowns Experiments\Results\21 KnockDown-siRNA-PKCa\Figures*