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Characterization of Select Lysine Mutations of the Cystine/Glutamate Transporter, System x_c^-

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Background: Oxidative Stress and System x_c^-

- System x_c^- functions in GSH synthesis by catalyzing cystine uptake which is used for glutathione synthesis (Fig. 1)
- Glutathione (GSH) is central to detoxification of hydrogen peroxide (H_2O_2) and protection from oxidative stress
- System x_c^- inhibition may induce ferroptosis, oxidative-stress dependent cell death, in cancer cells. It is also implicated in the pathology of neurodegenerative diseases such as Alzheimer's and Parkinson's Disease.

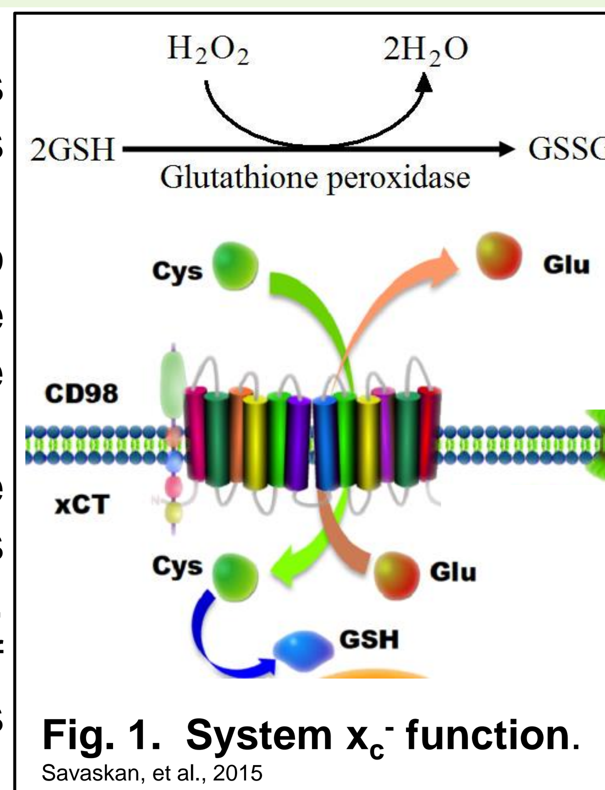

Fig. 1. System x_c^- function. Savaskan, et al., 2015

- Previous studies in the Chase lab have demonstrated that cells show significantly increased membrane localization of xCT following 10 min exposure to 0.3 mM H_2O_2 demonstrating that its activity is trafficking is regulated.

The goal of this project is to identify conserved residues within xCT that regulate its trafficking

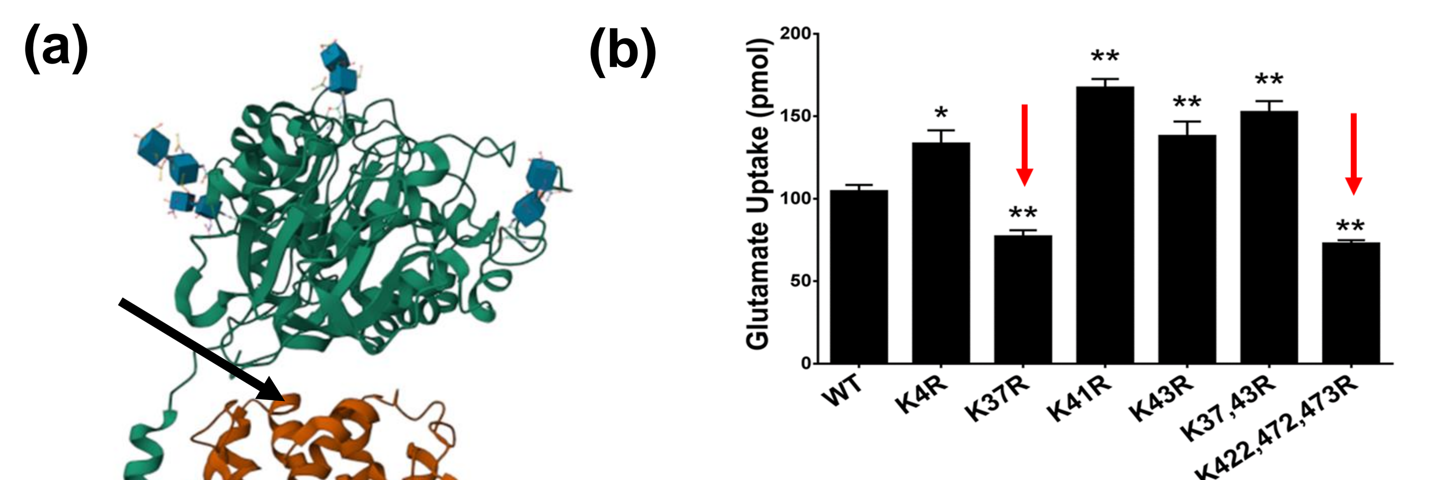

Fig 2. (a) Structure of System x_c^- made up of xCT (orange) and accessory protein 4F2HC (green) on the plasma membrane. Glycosylation site N314 (solid) and K472 and K473 (dashed) are shown. **(b)** K37R and K422,472,473R xCT mutants exhibit decreased glutamate uptake, suggesting they are less localized to the membrane.

- xCT is known to be ubiquitinated and contains several intracellular conserved lysine residues that may undergo post-translational modification

Methods

➔ We took a site directed mutagenesis-based approach to identify residues that regulate xCT trafficking. Mutants are then screened for function, changes in post-translational modification and localization.

➔ *Glutamate Release Assay* (function) COS7 cells transfected with xCT and 4F2HC were treated with 80 μM cystine and the released glutamate was measured using a glutamate oxidase/ horseradish peroxidase/Amplex red fluorescence assay.

➔ *Immunoprecipitation assay / Western Blot* was used to examine molecular weight shifts of xCT due to post-translational modification of the transporter.

➔ *Immunocytochemistry* allows for a semi-quantitative measure of co-localization of myc-xCT with the endoplasmic reticulum and qualitative assessment of membrane localization.

Results: K37R, K472R, and K473R exhibit diminished activity relative to WT, but only K473R exhibits a change in molecular weight relative to WT

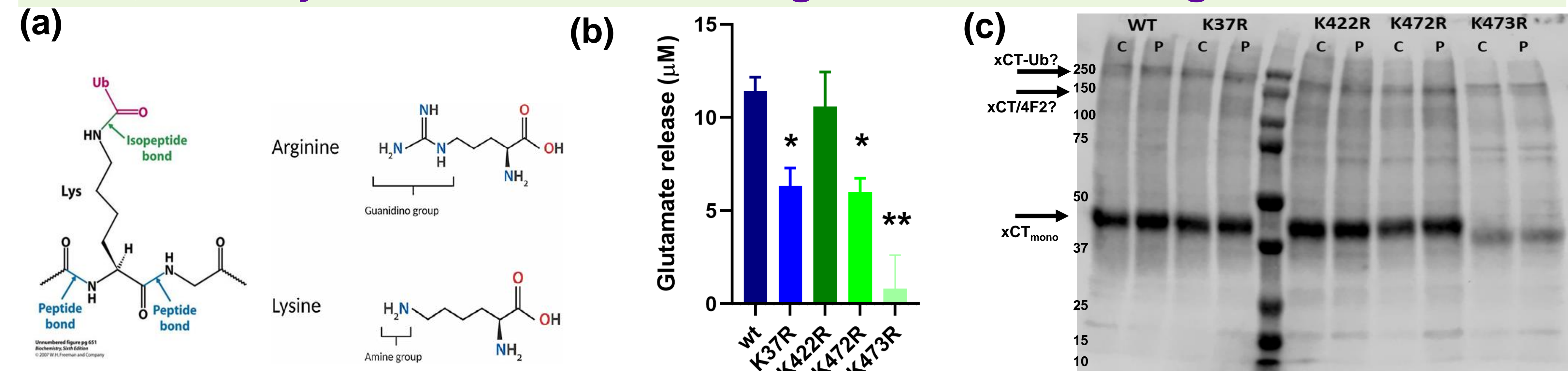

Fig 3. (a) Mutagenesis strategy. **(b)** Effect of mutation on transport activity using glutamate release assay * p<0.01, **p<0.001, **(c)** Molecular weight analysis of K37R, K422R, K472R and K473R. The most notable finding was a 7 kD shift in molecular weight for K473R in control (C) and peroxide (P) conditions.

Results: K472R and K473R exhibit fewer higher molecular weight complexes and K472R exhibits greater ubiquitination

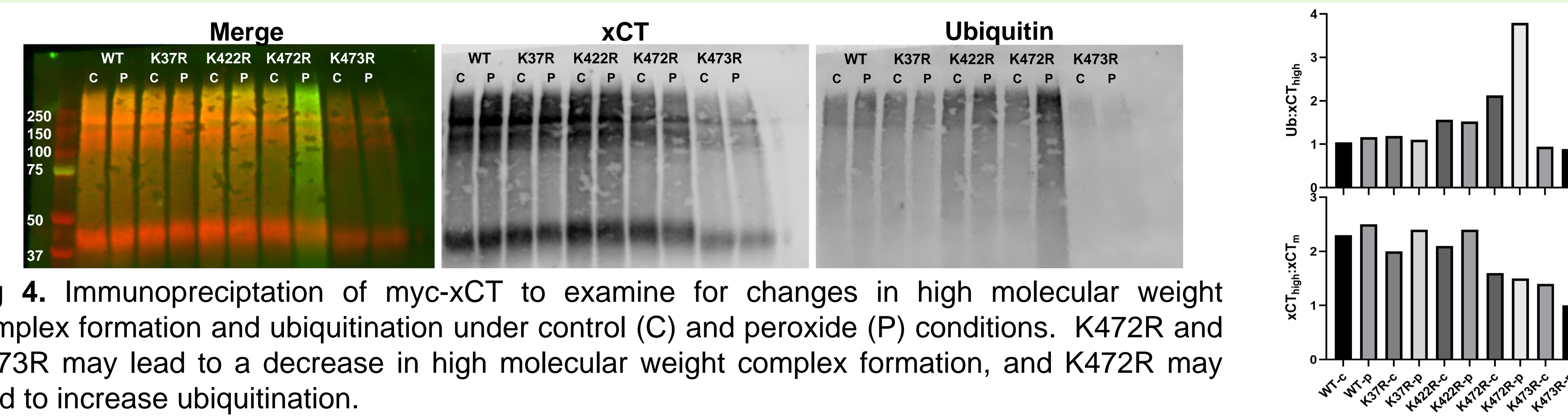

Fig 4. Immunopreciptation of myc-xCT to examine for changes in high molecular weight complex formation and ubiquitination under control (C) and peroxide (P) conditions. K472R and K473R may lead to a decrease in high molecular weight complex formation, and K472R may lead to increase ubiquitination.

Results: K473 appears to regulate N-glycosylation of xCT

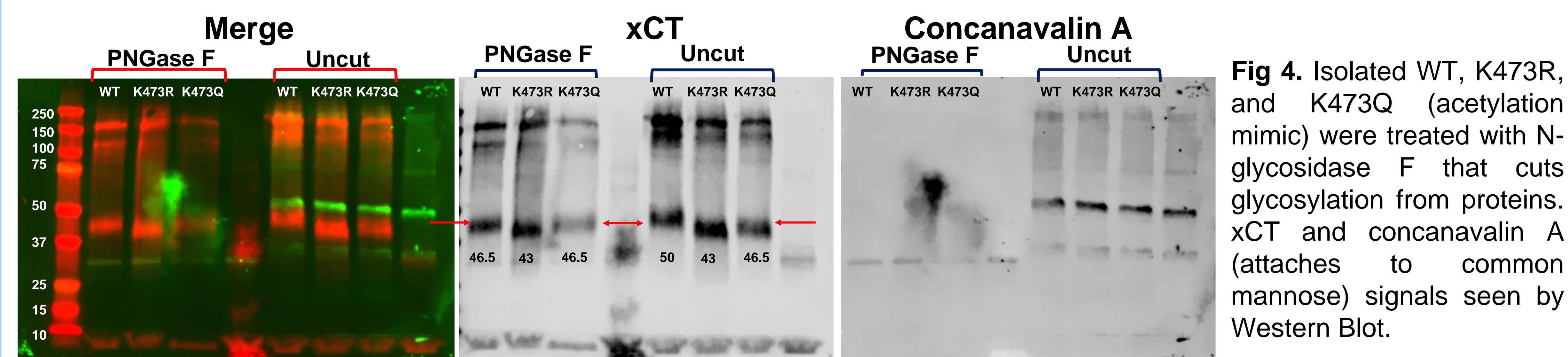

Fig 4. Isolated WT, K473R, and K473Q (acetylation mimic) were treated with N-glycosidase F that cuts glycosylation from proteins. xCT and concanavalin A (attaches to common mannose) signals seen by Western Blot.

Results: K473R and K472R show colocalization with endoplasmic reticulum and decreased localization on the membrane

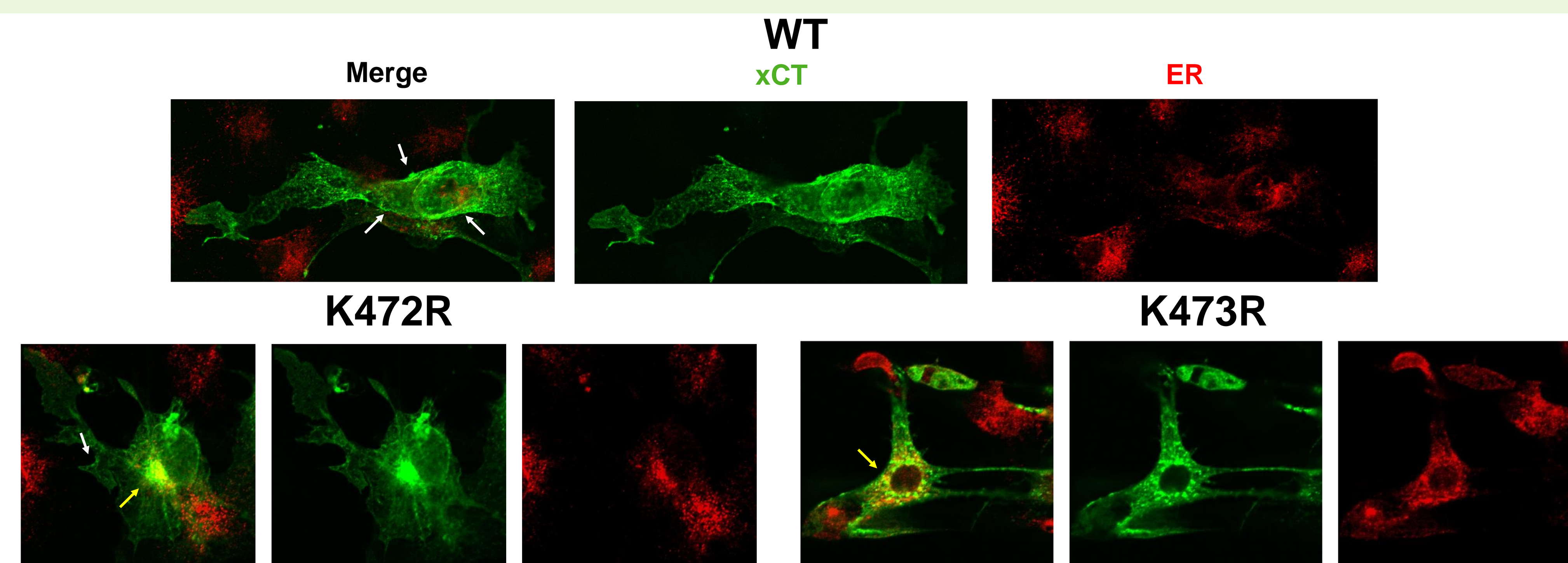

Fig 5. ICC images of WT, K472R, and K473R with xCT (green) and ER (red) signals marked. Yellow arrows indicate ER co-localization, white arrows indicate membrane localization.

Results: K37R and K472R show diminshed membrane expression

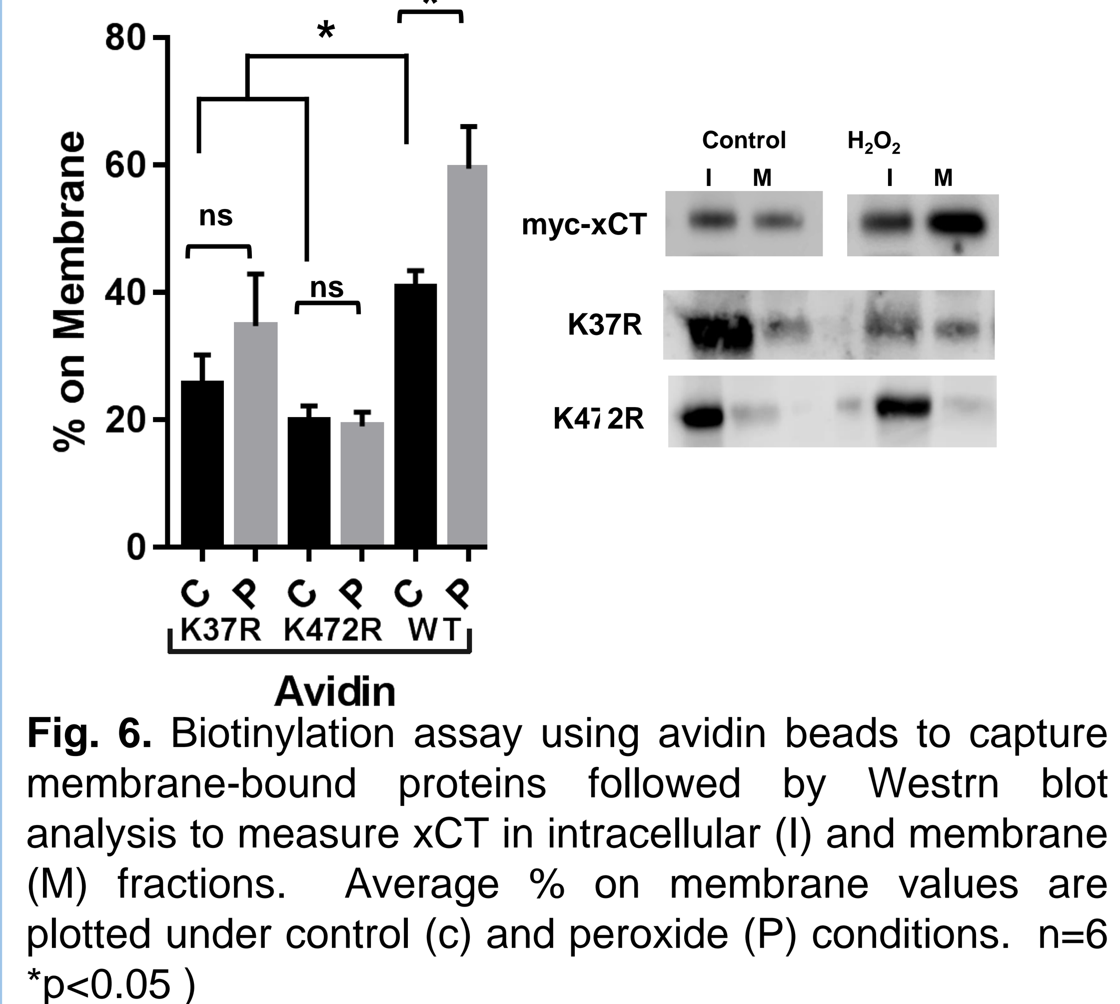

Fig. 6. Biotinylation assay using avidin beads to capture membrane-bound proteins followed by Westrn blot analysis to measure xCT in intracellular (I) and membrane (M) fractions. Average % on membrane values are plotted under control (c) and peroxide (P) conditions. n=6 *p<0.05)

Summary of Results

- K37R exhibits reduced membrane expression and activity, but we have yet to determine the PTM that is governing this regulatory site.
- K422R appears to have no effect on transport activity or on Ub of the transporter.
- K472R exhibits diminished activity and membrane expression. This may be a result of being more highly Ub. K472 may also regulate the exit of xCT from the ER or regulate its targeting to lysosomes/proteosomes.
- K473R exhibits a 7 kD reduction compared to WT. This may be in part N-linked glycosylation that governs its exit from the ER. An acetylation mimic at K473 leads to a 3.5 kD shift in molecular weight relative to K473R, but the nature of this PTM is not known at this time.

Future Plans

- Explore new methods for visualizing glycosylation.
- Study the effect of N314Q (N-glycosylation lacking mutant) on PTM, cellular localization and activity.
- Explore other potential PTM of xCT triggered by K473 acetylation
- Complete biotinylation experiments with all mutants
- Create K472Q mutant to better understand the role of K472 in regulating ER exit and membrane localization of xCT.

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