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Creation of an N-terminal xCT Mutant Lacking Lysines for Use in Protein Turnover Studies

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Creation of an N-terminal xCT mutant lacking lysines for use in protein turnover studies

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Background

Function of xCT

- System x_c^- is an antiporter that exchanges intracellular glutamate for extracellular cystine
- Cystine is reduced to cysteine, which is necessary for the synthesis of glutathione (GSH)
- GSH is a reducing agent that mitigates oxidative stress in cells
- System x_c^- consists of the light chain protein, xCT, that confers specificity, and the heavy chain protein, 4F2HC
- Trafficking of xCT is thought to modulate the oxidative stress response

Effects of Ubiquitin

- Ubiquitin tags on proteins typically leads to their internalization or degradation
- Ubiquitin reversibly attaches to lysine (K) residues
  - 6 K's are in the N-terminus of xCT: 4, 12, 30, 37, 41, 43. Four K's are conserved across species (4, 37, 41, 43). (Figure 1)
- Other proteins, such as epithelial sodium channel, are regulated by ubiquitin, with increased ubiquitination resulting in decreased membrane expression

(a)

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Human xCT    1 MVRKPVVSTI SKGGYLQGNV NGRLPSLGNK EPPGQEKVQL KRKV 44
Mouse xCT    1 MVRKPVVATI SKGGYLQGNM SGRLPSMGDQ EPPGQEKVVL KKKI 44
Zebra Finch  1 MFRKAAVPTV SNGSYLQGQA NGKLSSTDSG QPAREGKVVL KKKV 44
Zebra Fish   1 MPRRTVSAPH PNGDPTPSNF GEKEPLKLNG EPPKE-KVEL RKKV 44
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(b)

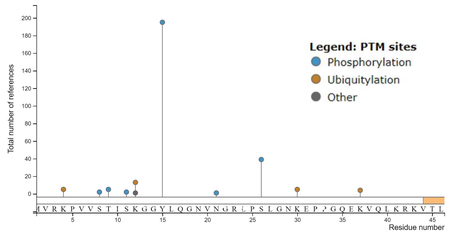

Figure 1. N-terminus xCT DNA sequences for four species. (a) A comparison of the DNA sequence of xCT for *Homo sapiens* compared to 3 other species. Most of these lysines are highly conserved across species. (b) Post-translational modifications of amino acids in the N-terminus of xCT identified through high-throughput screening (Phosphosite plus).

Research Question

How does the lack of ubiquitination on the N-terminus of xCT affect the function of the protein?

Hypothesis

Replacing all of the lysines in the N-terminus of xCT with arginines will lead to a loss of ubiquitination and an overall increase in the activity and overall expression of the transporter.

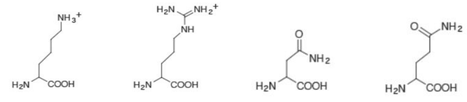

Figure 2. Mutagenesis strategy. Arginine and lysine are interchangeable because they are similar in size and charge, but arginine cannot be ubiquitinated, allowing for preservation of protein structure while allowing us to test the importance of ubiquitnation. Asparagine and glutamine are amino acids that are found in other species (besides humans) at positions for K. These can serve as acetylated mimetics of lysines.

Methods

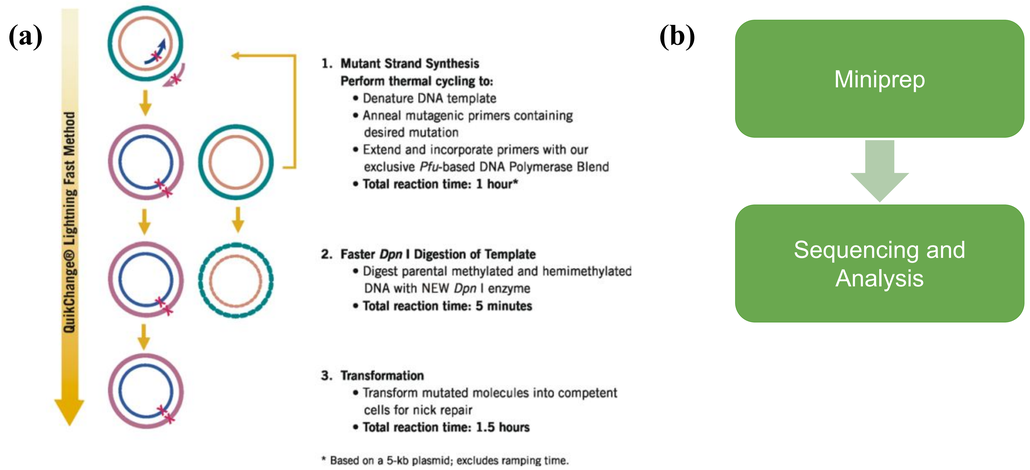

Figure 3. Schematic of overall experimental approach. (a) Process for inserting successive mutations in the N-terminus of xCT (QuikChange Lightning Manual, 2015). We started with K37,41,43R and successively replaced K's at the other 3 positions. **(b)** Following transformation, the samples were mini-prepped and sent to Eurofins for DNA sequencing.

Sanger Sequencing Trace for N-terminus of xCT

Figure 4. DNA Sequencing results of final 6K mutant. (a) Sanger sequencing trace of K4R and K12R on the N-terminus of xCT. A missense mutation occurred in both amino acids, changing Lys→Arg. **(b)** Sanger sequencing trace of K30R, K37R, K41R, and K43R on the N-terminus of xCT. In every case, the peak represented is guanine (G), which replaced adenine (A), leading to the change of the codon from AAA or AAG (lysine codons) to AGA or AGG (arginine codons), respectively.

Comparison of 6K xCT mutant amino acid sequence to native *Homo sapiens*

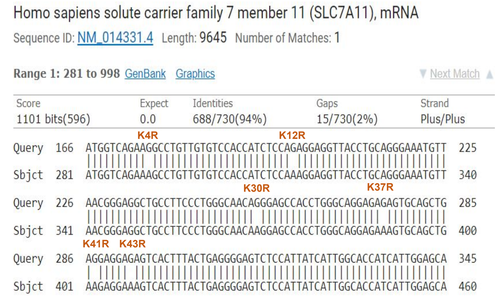

Figure 5. Nucleotide-blast for mutated xCT. A nonsynonymous SNP from A→G at positions 291, 315, 369, 390, 402, and 408 causes a missense mutation of Lys→Arg in K4R, K12R, K30R, K37R, K41R, and K43R.

Conclusions

- Previous work in the Chase lab has shown the replacement of individual lysines with arginines does not lead to obvious changes the ubiquitination of a transporter
- Currently, all 6 N-terminal lysines in xCT have been replaced by arginines.
- It is unknown if complete removal of lysines in the N-terminus will result in a lack of ubiquitination or change in transporter function or overall expression.

Future Directions

- Transfect COS-7 cells (mammalian cells) with the 6K xCT mutant to study its level of expression and function in cells
- Immunoprecipitate the 6K xCT from the cells and perform Western blotting to examine the uibqiuitination status of the 6K xCT mutant
- Examine the rate of turnover and cell surface expression of the 6K xCT mutant.
- If we find that xCT is still being ubiquitinated, investigate what other aspects of xCT may allow ubiquitin to bind to the protein.

Acknowledgments

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References

Lin, A., Hou, Q., Jarzylo, L., Amato, J. Gilbert, F. Shang, and H. Man. 2011. Nedd4-mediated AMPA receptor ubiquitination regulates receptor turnover and trafficking. *JNChem* 119:27-39.

QuikChange lightning site-directed mutagenesis kit instruction manual. 2015.