Long-term Expression of Cathepsin K Induces Unexpected Proteolytic Feedback to Maintain Proteostasis

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Introduction: Cysteine cathepsins are proteolytic enzymes that have increased production and activity by cells in tissue destructive diseases such as atherosclerosis, cancer, and osteoporosis, where they play integral roles in degrading extracellular matrix proteins. Pharmaceutical companies have recognized them as important targets and have been developing inhibitors, however, all but one of the 16 drugs have been discontinued from clinical trials, mostly due to side effects. There is a need to understand why these well-designed inhibitors have not been deployable *in vivo*. Our lab's previous research has demonstrated that some cathepsins will preferentially degrade other cathepsins, even in the presence of target substrate, a behavior we termed *cathepsin cannibalism*. Cathepsin S (catS), for example, is known to degrade catK over collagen and is hypothesized to also degrade catL. Determining cathepsin cannibalism directionality and substrate preferences will begin to unravel the complex proteolytic network dynamics of these enzymes, and provide insight into their levels *in vivo* that complicate pharmaceutical dosing and inhibition strategies. These studies may also help elucidate cellular feedback responses to produce other proteases that can interact and change the amount present in complicated networks. I hypothesize that stable expression of catK to mimic diseased cells will induce co-regulation of other proteases.

Materials and Methods: Putative cleavage sites were determined bioinformatically for cathepsin K (cat K) and mutations were introduced using site-directed mutagenesis into a pcDNA4/TO/myc-His vector. HEK293T cells were stably transfected for overexpression of wildtype or mutant cat K. To test for activity, cathepsin mutants were assayed via multiplex cathepsin zymography, a modified SDS-PAGE method to image active cathepsins after renaturation in polyacrylamide gels. HEK293T cells stably transfected to express catK mutants were then transiently transfected with cat S to study cellular responses to additional perturbations to the cathepsin proteolytic network. Furthermore, the network was perturbed with E-64 a broad spectrum cathepsin inhibitor to block proteolysis among cathepsins.

Results and Discussion: Initially, at week 0, cathepsin K (catK) mutants showed expression and activity of catK and catL. After 2 weeks of culture, catK activity and expression was lost, while catL activity was preserved. Loss of cat K activity suggested cells upregulate cat L in response to stable expression of catK. In an attempt to recover catK signal, cells were forced to express catS, hypothesizing cat S would degrade catL, so catL could not degrade catK. Activity shows a ~30 kDa band, uncharacteristic of the 25 kDa activity band catS traditionally shows. The non-reduced western blot detected 3 catS bands: the expected 37kDa pro-form, 25 kDa mature form, and a ~30 kDa band similar to the zymography, suggesting a protease complex was formed between catS and K. Inhibition of all cathepsin activity with E-64 showed recovered catK expression comparable to freshly cultured cells.

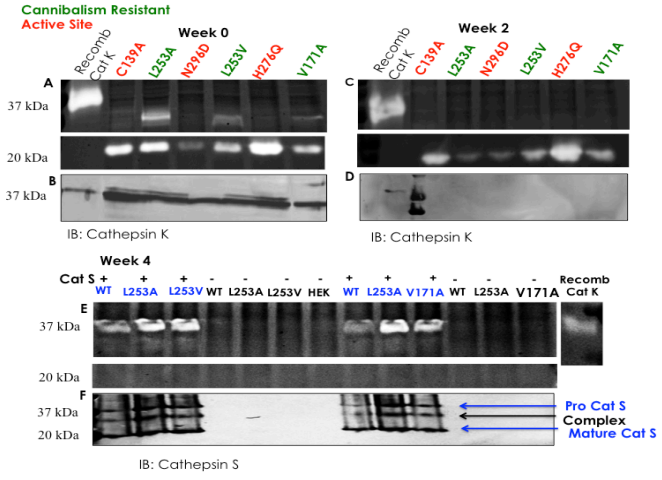

Figure 1: Cathepsin K activity is lost in stably transfected cells after 2 weeks of culture, and transient transfection of cathepsin S was performed to recover K activity. Week 0 zymography shows cat K (37kDa) and cat L (20kDa) activity in mutants (A). Western blot shows cat K expression (B). Cat K activity (C) and expression (D) are lost while cat L activity (20 kDa) (C) is preserved after 2 weeks of culture. Zymography shows mutants transiently transfected with cat S have a strong active S band at ~30 kDa (E), while cat L activity is lost in all samples compared to stably transfected cells. Non-reduced western blot detected 3 cathepsin S bands: 37kDa pro-form, 25 kDa mature form, and a ~30 kDa cat S complex band (F).

Conclusions: Long-term expression of cathepsin K (catK) causes co-regulation of other proteolytic enzymes. Specifically, HEK 293T cells upregulate catL in response to stable expression of catK. CatS can form a complex with other proteases, likely catK or L and remain active; suggesting the active site of catS is unaffected. Small molecule inhibitor E-64 protects against cathepsin cannibalism, suggesting initial loss of catK activity was due to cathepsin cannibalism. These proteolytic interactions and co-regulation of these proteases could begin to explain the unexpected side effects of the pharmaceutical cathepsin inhibitors.