The Role of Ubiquitination in Ras Signaling and Activation

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33% of cancers are due to Ras mutations. As an essential protein inside the cell, Ras is involved in cell regulation, development, and death. It functions as a signaling switch that turns itself on and off. The role of Ras is to turn itself off, so that it stays in control, does not have unregulated cell development, and prevents cancer. As an enzyme, Ras acts slowly, so there are regulators that speed up the process. The molecule that turns it off is GTPase activating protein (GAP), and the molecule that turns it on is guanine nucleotide exchange factor (GEF). When Ras is turned on, it is bound to GTP and turns off when the GTP is hydrolyzed to GDP. Ras can be stuck in an on position for much longer than necessary when it does not respond to GAP. This is what leads the cell to becoming cancerous.[1] Recent evidence, however, suggests that post-translational modifications of Ras may also be important for Ras activation and regulation. Since targeting Ras directly in cancer treatments has been historically challenging, this observation offers an alternative approach to thinking about treating Ras-driven cancers. The post-translational modification in which we are interested is ubiquitination, the modification of a protein with the 8 kDa protein ubiquitin. Current literature evidence suggests that Ras ubiquitination leads to increased activity and improves binding to downstream effectors.[2]

Our goal as a lab is to better understand the role ubiquitination plays in the regulation of Ras signaling. Mammalian cells are difficult to work with, therefore we chose a simpler model; yeast. Yeast and mammalian cells are similar enough that the results we obtain from working with yeast can be applied to work with human forms of Ras. This will give us a better understanding on how to approach finding an alternative treatment for Ras-driven cancers. My goal is to modify Ras *in vitro* with ubiquitination to understand how it alters the activity of Ras. The addition of a single ubiquitin is called monoubiquitination. My goals for this summer were to (1) purify yeast Ras proteins, (2) characterize activity of yeast Ras *in vitro*, (3) measure binding to the downstream effector Cyr1, and (4) determine impact of ubiquitination on activity.

My first goal was achieved last summer, but it needed improvements. The new purification protocol that I developed worked ten times better than last summer. The protein was originally washed in a buffer with a high salt concentration. We found out that the protein would not stick to the column very well and was not being collected. After realizing this, the high salt buffer was removed and replaced with a low salt wash buffer and the results improved tremendously. A detergent was also added to the protocol which helped the protein stay soluble in my solution. I was able to purify enough protein to last for months.

My second goal was to characterize the activity of yeast Ras and that was done through two different assays. The first was a nucleotide exchange assay. The sample of Ras protein was loaded with MANT GTP, which would fluoresce so that a signal could be measured. The goal was to be able to measure the decline of signal as GTP dissociated and became GDP. As we analyzed the results, we found something surprising. When Ras 2 is lacking the c-terminal tail, it shows an increased rate of nucleotide exchange. Our next steps are to use circular dichroism (CD) to look for differences in stability and then to ubiquitinate Ras to observe changes in activity. We are currently completing our analysis of nucleotide hydrolysis as well and are eager to see if this same pattern is repeated.

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[1] Wennerberg, K., Rossman, K. L., and Der, C. J. (2005) The Ras superfamily at a glance.J. Cell Sci. **118**, 843–846

[2] Hobbs GA, Gunawardena HP, Baker R, Campbell SL. Site-specific monoubiquitination activates ras by impeding gtpase-activating protein function. Small GTPases. 2013;4:186–192.

My third goal was to measure the binding of Ras to downstream effectors through a pull down experiment. Cyr1 is a downstream effector that interacts with Ras. I performed a Pull Down experiment to show that Ras binds more efficiently to Cyr1 in the presence of GAP.

To address my fourth goal, I am currently completing mass spec studies to verify ubiquitination and setting up *in vitro* ubiquitination assays.

These four goals were designed to help us create an understanding of how the ubiquitination of Ras can be used to target cancer. When Ras is insensitive to GAP, it leads to a cancerous state. When it is insensitive, its activation is prolonged and triggers an uncontrolled downstream cascade through the binding of Cyr1. Monoubiquitination can be a target to treating cancer. The monoubiquitination increases the activity of Ras and would stop the uncontrolled downstream cascade. The activity is increased because GTP loading is enhanced and that turns Ras on. The information we obtain from our yeast studies will provide insights to further research for human forms of Ras. Therefore, if we can develop a better understanding of how ubiquitin plays a role in yeast Ras signaling, we can apply it towards a target for cancer.