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Impact of ROS Presence on Oncogenic Ras Activity

Chris Andersen
University of Puget Sound, chrisandersen@pugetsound.edu

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Chris Andersen*, Dr. Leslie Saucedo
Affiliation: University of Puget Sound

Department Address/Contact Information:
1500 N Warner St, Tacoma, WA 98416,
chrisandersen@pugetsound.edu

• Abstract:

Previous research has suggested a connection between oncogenic Ras and the cell's levels of Reactive Oxygen Species (ROS).[1] The underlying cellular mechanism is not well understood. To investigate this connection, we applied the UAS-GAL4 system in *Drosophila melanogaster* flies to control the expression of Ras and Keap1, a key redox regulator.[2] We expected the activity of Ras to vary with its redox environment and thus impact protein activity downstream of Ras signaling cascades. In monitoring three proteins downstream of Ras—Dcp-1, Akt, and MAPK—we aimed to determine which pathways were impacted by ROS modulation.

• Background:

UAS and GAL4 are gene regulators that, when both are present, overexpress a gene of interest within the genome.[3] When Keap1 is overexpressed, we expect cellular ROS levels to increase dramatically. To decrease ROS levels, we apply an interfering RNA sequence to Keap1, denoted Keap1^{RNAi}, to prevent Keap1 transcription.

Previous work in Dr. Saucedo's lab has shown that overexpressed Ras leads to fly death and that either increasing or decreasing ROS levels via Keap1 with overexpressed Ras rescues all flies from lethality. This suggested that multiple pathways are involved in rescuing the flies from Ras, either by halting cellular division, increasing the rate of apoptosis, or a mixture of both. Dcp-1 and Akt are typically involved in apoptosis signaling while MAPK is involved in cellular proliferation.

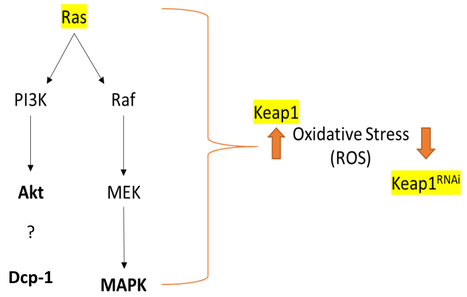

Figure 1. Targets of the Ras Pathway. In this simplified version of the Ras pathway, there are two main routes that protein signaling can take. We can examine the activity of Akt, Dcp-1, and MAPK to determine how Ras behaves in response to varying levels of oxidative stress via Keap1 and Keap1^{RNAi}.

Objectives:

- To identify which pathways are affected by Ras activity and lead to adult, organismal death
- Propose a mechanistic relationship between ROS and Ras

Methods:

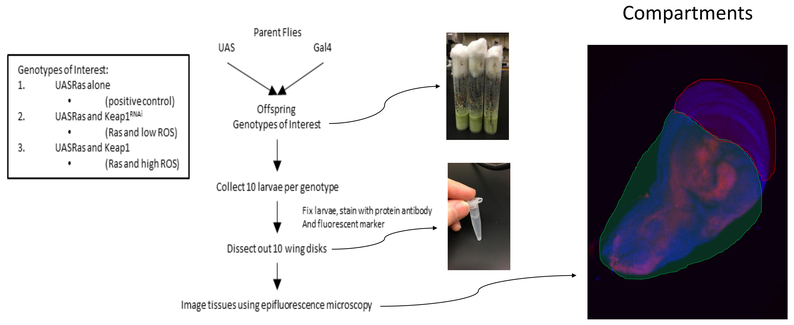

Figure 2.Procedure summary. The sampling process begins by crossing flies with the UAS transgenes of interest with Gal4 flies. When the offspring reach the larval stage, they are collected, dissected, and stained using an antibody for Dcp-1, Akt, or MAPK. Once the antibody staining is complete, the wing disks are isolated and mounted onto slides and imaged using conventional epifluorescence microscopy. Once the images have been taken, each wing disk is separated visually into two regions, dorsal and ventral, separated above visually by the white bar. ApGal4 expression only occurs in the dorsal region, so data based on pixel count and intensity is recorded for each region of every wing disk.

When the offspring reached adulthood, they were sorted by their phenotypes. Each cross was set using a known combination of dominant markers which, if inherited, exhibit a known phenotype. One such marker was Green Fluorescent Protein (GFP), and a sample cross is shown below (Figure 3). Offspring, adults and larvae, were screened regularly for each marker to determine their genotypes.

	Parent 1: GFP	Parent 1: Gal4
Parent 2: GFP	Offspring 1: GFP/GFP	Offspring 2: GFP/UASRas
Parent 2: UASKeap1	Offspring 3: UASRas/GFP	**Offspring 4: UASKeap1/Gal4**

Figure 3. Sample Cross of Dominant Marker. Two parents heterozygous for GFP/UASKeap1 and GFP/Gal4 are crossed. Offspring 1-3 will fluoresce green when exposed to certain light, however offspring 4 will not. Selecting against GFP, therefore, yields all offspring with overexpressed Keap1.

• Results:

No adults survived with overexpressed Ras alone (control) or Ras and Keap1 (high ROS). Adults did survive, however, with overexpressed Ras and Keap1^{RNAi} (low ROS).

All wing disks were stained for the protein of interest (red) and DAPI (blue)– the latter stains in the nucleus and visually represents the total size of each wing disk (Figure 4-6). Each graph below shows the ratio between all red signals within the dorsal region, where apGal4 is expressed, and the ventral region, where apGal4 is not expressed. A value greater than 1 indicates increased expression of the protein of interest due to apGal4 expression.

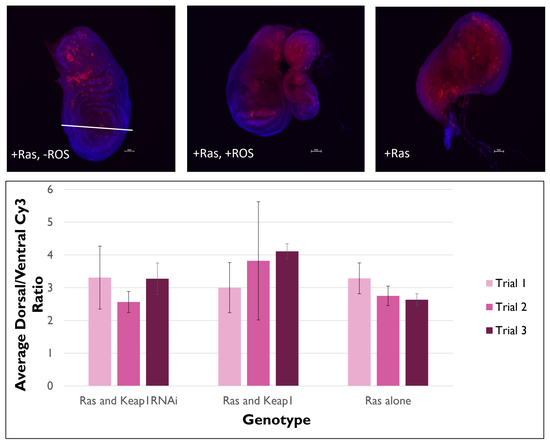

Figure 4. Antibody Staining Results for Dcp-1. Dcp-1 expression was increased for all genotypes due to apGal4, but no significant pattern emerged. Dorsal and ventral compartment separation in +Ras, -ROS indicated by white bar.

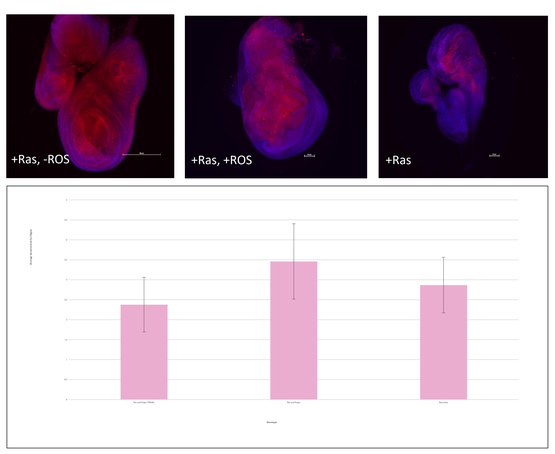

Figure 5. Antibody Staining Results for Akt. Only one trial was completed for this antibody due to limited antibody supply, however the staining ratios remained consistent with Dcp-1 results summarized above. No significant pattern emerged.

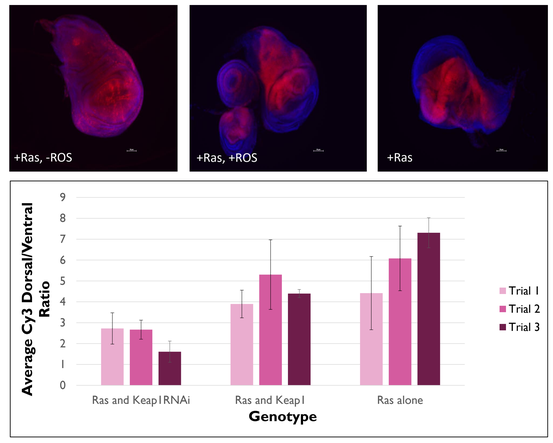

Figure 6. Antibody Staining Results for MAPK. Significant decrease (p-value = 0.00094) in MAPK expression appeared in all three trials for the Ras and Keap1^{RNAi} genotype. While this expression still remained greater in the dorsal than it did in the ventral compartment, the expression was still lowerin comparison to that of the control.

• Conclusions:

Flies with overexpressed Keap1^{RNAi} and Ras survive to adulthood and, while in the larval stage, exhibit significantly lowered MAPK activity in the dorsal region of their wing disks. Neither result was observed in flies with overexpressed Keap1 and Ras or in the control. Therefore, only a decrease in ROS is capable of rescuing fruit flies from death due to Ras overexpression. This decrease in ROS is reflected in lowered MAPK activity, indicating that cell proliferation rates decrease as a result of heightened ROS regulation, thus allowing flies to evade cancerous Ras activity and survive to adulthood. While the Dcp-1 and Akt trials were relatively incomplete, the staining suggests that modulating ROS levels does not impact the Akt side of Ras signaling.

• Future work:

In only manipulating Keap1, there is no direct or indirect way to measure ROS in the cells of interest to confirm that ROS levels are in fact being affected by Keap1 expression. We attempted to stain for ROS using dihydroethidium (DHE), however this approach did not yield significant results as the procedure requires further optimization. Future work may attempt to optimize the DHE procedure or measure with a different redox probe.

To confirm that these results hold true in other cellular contexts, another future direction may be to repeat this method in other tissues. ROS levels are expected to be high in larval wing disks since cells are dividing frequently. Confirming these results in tissues with lower average ROS production may be particularly informative in confirming these results.

• References:

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2. Taguchi, K., & Yamamoto, M. (2017). The KEAP1–NRF2 System in Cancer. *Frontiers in Oncology*, 7. https://doi.org/10.3389/fonc.2017.00085
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