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Headcase Regulates Growth in Response to Nutritional Status Downstream of Insulin Signaling

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Headcase Regulates Growth in Response to Nutritional Status Downstream of Insulin Signaling

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Abstract

Cancer cells are notorious for growing in an unrestricted manner without regard for environmental cues. Recently, Li et al. (2019) discovered headcase (hdc) functions by binding to the mTORC1 complex in the mTOR signaling pathway and preventing further signaling. Interestingly, under nutrient restricted (NR) conditions, cells with mutated hdc proteins proliferated more than cells with normal functioning hdc. It is well known that insulin signaling is downregulated under NR conditions, so a potential signaling pathway with insulin, PI3K, PDK1, Akt, PTEN, and hdc was created as a way to explain the link between hdc function and nutritional status. A *Drosophila melanogaster* model using UAS-Gal4-induced eye-specific insulin signaling downregulation and Flippase-FRT-derived mosaic expression of mutant hdc cells and GFP wildtype (WT) cells was created to test this hypothesis. The eye discs of the third instar larvae were dissected, and observed under a fluorescent microscope. Hdc mutant clone sizes were the same as WT clones under normal insulin signaling conditions. However, when insulin signaling was downregulated, hdc mutant clones did tend to amass more total area than WT clones. Due to variability in clone sizes within eye discs, inadequate clone sizes for comparison in some eye discs, and lack of sampling, these observations are not statistically proven. Thus, this insulin signaling pathway shows some potential to exist. Further data collection and improvements on the model need to be pursued in order to come to more concrete proof about the existence of this insulin signaling pathway.

Keywords: Drosophila, mTORC1, PTEN, InR, insulin signaling, tissue growth

Introduction

Cancer is a very devastating disease. As per National Cancer Institute (2020), there will be about 1,806,590 new cancer diagnoses in 2020, with an estimation of 606,520 cancer-related deaths. Cancer is caused by cells in the body becoming mutated, bypassing normal cell cycle checkpoints, and growing in an unrestricted manner. These fast-growing cells compete with and kill surrounding tissue, eventually gaining access to the cardiovascular system through processes such as angiogenesis, and metastasize to other parts of the body.

A key pathway that is involved in the metabolic and proliferative differences between cancer and normal cells is the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway regulates the genetic expression and protein synthesis that controls cell proliferation, immune cell differentiation, and metabolism. There are two protein complexes that are formed with mTOR in the mTOR signaling pathways, mTORC1 and mTORC2. mTOR, GβL, deptor, and raptor form mTORC1 and Rictor, deptor, mTOR, SIN1, GβL, and PRR5 form mTORC2. mTORC1 controls cellular metabolism by synthesizing a response from various growth factors, nutrients, and signals of energy supplies. These responses are either cell growth when there is plenty of nutrients or catabolism when nutrients are lacking. mTORC2 mostly controls cell proliferation and survival. In tumor cells, the mTOR pathway is abnormally activated, allowing tumor cells their characteristic ability to rapidly proliferate and spread (Zou et al., 2020).

mTORC1 is regulated upstream by PI3K, AKT, and PTEN. Mutations in PTEN, as well as changes that activate the PI3K/AKT/mTORC1 pathway have been shown to aid tumor proliferation and survival in cancers such as colon cancer (Zou et al., 2020). According to Nowak et al. (2013), downregulation of PTEN or upregulation of PI3K/Dp110 signaling can cause overproliferation of mutated cells at the expense of wildtype cells. This overproliferation

was proven to happen mainly through protein kinase B (Akt) and mTORC1 activity (Nowak et al., 2013). As per Li et al. (2019), the proteins Headcase (Hdc) and Unkempt (Unk) were shown to regulate cell growth by binding to Raptor in the mTORC1 complex and preventing the progression of the signal for cell growth.

When Hdc and Unk were mutated to be non-functional, the organism grew larger (Li et al., 2019).

The *Drosophila melanogaster* model organisms used in Li et al. (2019) were exposed through a diet that causes nutrient-restriction without malnutrition (NR), where the organisms were exposed to less protein than a normal diet (5 g/L versus 20 g/L yeast). Under the NR condition, the clones (groups of cells) expressing mutated *hdc* or *unk* were shown to cover more area compared to regular cells in both the eye discs and the wing discs. The *hdc* and *unk* mutant-expressing clones were also shown to proliferate more via increased pS6 signaling (Li et al., 2019). pS6 is a ribosomal protein that is upregulated and phosphorylated via Akt/mTORC1 activation and assists with cell proliferation (Meyuhas, 2015). However, under normal diet conditions, there is no difference between *hdc* or *unk* clones and wildtype (WT) clones in terms of clone sizes or proliferation.

Abnormalities in HECA, the human equivalent of Hdc, have been noted in lung, neck, head, pancreatic, and renal cell cancer (Li et al., 2019). Elevated levels of HECA have led to good prognosis for patients of estrogen receptor-positive breast cancer (Wang et al., 2020). HECA can also slow down head and neck cancer cell mitosis (Dowejko et al., 2009). As the Human Protein Atlas, HECA generally had low or negative expression in many cancer tissues such as melanomas, lymphomas, and prostate cancers.

PTEN, which negatively regulates PI3K activity, was shown to help cells in *D. melanogaster* larvae overproliferate in its mutated form. The overgrowth was shown to happen strictly via Akt/PKB and mTORC1 signaling.

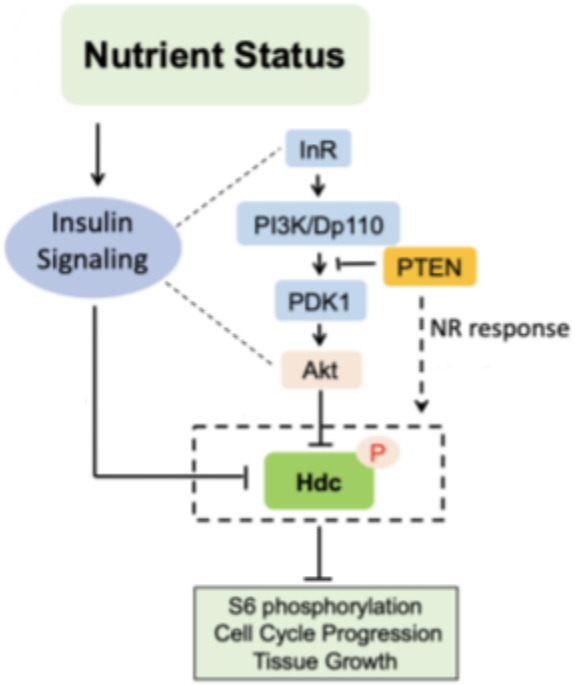
Based on Li et al. (2019), there is a clear connection between Hdc function and nutritional status. An interesting point is that during NR, many growth factors, especially insulin-like growth factors (IGFs) and insulin, are decreased. Insulin and IGFs are shown to signal with insulin receptor (InR), which binds to phosphatidylinositol 3-kinase (PI3K) in order to phosphorylate phosphatidylinositol to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 causes phosphoinositide-dependent protein kinase 1 (PDK1) to activate protein kinase B (AKT) (De Meyts, 2016; Regan et al., 2019). PI3K/AKT signaling is shown to interact with mTORC1. Thus, it was hypothesized that insulin signaling via the INR/PI3K/PDK1/AKT pathway allows Hdc to inhibit cell growth based on the nutrition the organism receives (Figure 1).

In order to test this InR/hdc signaling pathway, a model was created using *D. melanogaster* or fruit flies. Aside from the fact that many of the other papers present a lot of information using this model for easy comparison, up to 75% of genes implicated in human diseases have homologs in the *D. melanogaster* genome. Additionally, fruit flies make an ideal model organism to study human diseases because they require very little to maintain, have a ten day generation cycle, have low genetic redundancy, and have only four chromosomes.

This paper will delve into the effectiveness of this model in discovering the connection between nutritional stress and *hdc* function.

Figure 1

Insulin Signaling Pathway



Note. This is the proposed insulin signaling pathway that connects hdc function to nutrient status signaling.

Results

No Proliferative Advantage of *hdc*$^{\Delta}$ over WT Clones in Normal Diet

When comparing the size of the *hdc*$^{\Delta}$ clones to WT clones in the control cross (no downregulation of insulin signaling pathway), the area the *hdc*$^{\Delta}$ clones and WT clones cover is relatively the same (Figure 2A). While this stands true for the most part, there is some variation in clone sizes that make it somewhat difficult to truly state this observation to be true of the vast majority.

InR^{K1409A} expression Effect on *hdc*$^{\Delta}$ Clone Size

InR^{K1409A} is a dominant-negative allele of the insulin receptor. When InR^{K1409A} was overexpressed, the overall area of the hdc^{Δ} clones seemed to be larger than WT clone total area (Figure 2B). However, many of the hdc^{Δ} clone areas were scattered across the eye discs as smaller clones. In some cases, the hdc^{Δ} clone proliferative advantage by size over WT clones can be seen, but the clones are too small to present as a decent comparison. Many of the eye discs lacked hdc^{Δ} clones, so hdc^{Δ} to WT clone comparisons are difficult to make. In terms of individual clone sizes, some hdc^{Δ} clones were bigger than adjacent WT clones while others were smaller than WT clones.

$Dp110^{D954A}$ expression Effect on hdc^{Δ} Clone Size

$Dp110^{D954A}$ is a dominant-negative, kinase-dead version of Dp110 (PI3K) with the mutation in the putative ATP binding site. When $Dp110^{D954A}$ was overexpressed, the overall area of the hdc^{Δ} clones seemed to be larger than WT clone total area (Figure 2C). While there was some variation in clone sizes between eye discs, many more $Dp110^{D954A}$ eye discs had clones to allow for comparisons than were present InR^{K1409A} eye discs samples. However, there were still many eye discs where the clones were too small to make a decent comparison. Even within many eye discs, the individual clones sizes were either bigger or smaller compared to the wild-type clones.

PTEN overexpression Effect on hdc^{Δ} Clone Size

PTEN is a negative regulator of PI3K. PTEN overexpression has caused hdc^{Δ} clones to cover a greater area than WT clone sizes (Figure 2D). However, this difference in overall clone area coverage is much milder than seen in $Dp110^{D954A}$ and InR^{K1409A} eye discs that can be used for comparison, which could indicate that PTEN expression may not be as strongly interact with hdc as InR and Dp110 does. The same large amount of variation in clone size exists as in $Dp110^{D954A}$ and InR^{K1409A} expressing eye discs. Like $Dp110^{D954A}$ slides, there were more PTEN eye discs with

comparable clones per each slide than InR^{K1409A} slides. There are also many eye discs with clones too small to use for proper comparison.

Discussion

There are many improvements that can be made to this experiment and possibly this model. One issue about this experiment was that not many slides were created. In order to have enough slides for proper comparison, there needs to be at least five slides created for each cross. Concerning this most recent heat shock schedule, only three slides were made per each cross. Around 40 or more larval heads were dissected for the eye discs in order to make each slide. The dissections of these eye discs were mostly performed well so as to not destroy samples. Despite this, a few more slides per each of the four crosses would immensely help with the comparisons. Thus, these results point to the potential that this pathway exists, but there is not enough sampling to statistically back this claim.

The first potential improvements are the heat shock schedules. Initially, the larvae were heatshocked on a 24-48 hour schedule rather than the 24-36 hour schedule mentioned in the methods section. While the 24-48 hour heat shock schedule yielded many eye discs that did display hdc^{Δ} clones, those clones were not big enough to make a decent comparison, similar to the situation seen in many of the eye discs in this current experiment.

Previously, a version of the model which expressed flippase in the eyes using the eyeless promoter (eyFlp) rather than using a heat-inducible flippase (hsFlp) was attempted. The results were similar to that of the 24-48 hour schedule results.

Thus, the 24-36 hour schedule was adopted with hopes of causing the creation of bigger, more discernible clones. By heat shocking earlier in the larval stage than previously, more cells could arise from the ones affected by the heat shock event and induced to become hdc^{Δ} clones. In

terms of the amount of time used to heat shock the larvae, two hours was decided upon because this was the most amount of time that can be used to induce hsFlp activity. Any more time past two hours will have little to no effect on hsFlp activity than is already present within those two hours. Compared to the 24-48 hour and the eyFlp model, there were more comparable clones in the eye discs produced using the 24-36 hour model.

Another untested portion of the current working model is Akt. Although preliminary data shows that Akt interacts with and regulates *hdc* activity by phosphorylating it, the growth advantage hdc^{Δ} clones may possess over WT clones when Akt is down-regulated is not actually proven. Thus, this section of the insulin signaling pathway needs to be confirmed in order to truly say that the insulin-hdc pathway seen in Figure 1 is true.

One factor that prevented further research into this model were the several mishaps that happened along the way. These consisted of minor issues such as the crosses not being able to generate and lay as many eggs as normal, as well as dissecting the third in-star larvae from vials where the majority of larvae have already pupated. These issues were fixed in laters runs of this experiment; the final iteration of this experiment was performed without any of these afore-mentioned flaws.

There can be some improvements that can be made to the experimentation of this model. The amount of food present in the vials could be maintained at the same levels. While there were instances in the past where vials with significantly greater or lesser food had to be used, this was not so much an issue in this current iteration of the experiment but could be improved upon ad infinitum. The amount of time allowed for larval growth before heat shock could be better ascertained and possibly decreased. While it is known larvae typically hatch within one day, finding a way to have a larger majority of them hatch and go through the life cycle at the same

time would assist with timing the heat shock protocol and dissection timing (Roote et al., 2013). For example, while the majority of the eggs laid hatched by the time the vials were heat-shocked, there were still a small but sizable portion of the eggs that were not hatched by that time. While the heat-shocking procedure can still produce clones in unhatched fly larvae, knowing when the most amount of the larvae hatch is would optimally time the heat-shocking schedule. Another important consideration is that while heat shocking the vials sooner can potentially create even bigger clones that can be used for comparative statistics, there is a chance that heat shocking too soon can kill many of the eggs laid by causing recombination in too many cells that may result in lethal developmental effects.

Another way to better ensure the model works is by allowing more time to pick the flies for the crosses. The virgin females and the males chosen for the crosses were originally meant to be long bodied. However, flies with these recessive phenotypes appeared very rarely in the original parent stocks, which these selections were not made. However, choosing for these recessive phenotypes in the breeding flies from these stocks would have helped in creating more long-bodied larvae among the progeny of the crosses, thus allowing for better ability to dissect more larvae and make more slides.

One flaw with the heat shocking model is that the environmental setting that creates the hdc^{Δ} clones (heat shocking) occurs relatively later in the larval developmental stage compared to the original NR model. In the NR model, the lack of protein was present in the environment of dissected larvae as soon as the larvae hatched from the eggs. In comparison, the larvae in this experiment were heat shocked a while after being born. Thus, the hdc^{Δ} clones in this model were not exposed to the hypothesized down regulated insulin signaling as early as the hdc^{Δ} clones in the NR model. In order to circumvent this issue of inadequate exposure of hdc^{Δ} clones to a

situation mimicking the NR model, RNAi inhibition of the proteins in the proposed insulin signaling pathway could be attempted, as well as insulin signaling antagonists present in the food can be used. For example, some insulin signaling inhibitors to consider are GSK19045297 and Wortmannin, which inhibit InR and PI3K respectively (Huang et al., 2019). Also, antagonists mixed with the food may be a more time-feasible method than having to create fly lines that have the genetic machinery needed for RNAi inhibition.

Another potential direction for this model is antibody staining. In Li et al. (2019), pS6 antibody staining, Brdu labeling, and PH3 antibody staining were used to elucidate the rate of cell proliferation (pS6 staining) as well as the stages at which these cells were most likely to be in (Brdu labeling and PH3 staining). Brdu labeling targets cells in the S phase and PH3 staining targets cells in the M phase. While pS6 staining in the preliminary 24-48 hour heat shock model did point to the potential for increased signaling for cell proliferation in hdc^{Δ} clones, no such staining was done for this model. Thus, all these stainings could be attempted.

There are some other types of data that could also be collected. If enough slides are created, perhaps a comparison of eye disc size between the insulin-downregulated larvae and the control larvae could be performed. It is very difficult to dissect the eye discs in their entirety, so a potentially more feasible version of this would be to compare the size of the posterior sections. Also, it may help to let some of the long-body larvae develop into flies in order to compare overall fly size. These comparisons of overall eye disc and adult fly body size would prove that downregulation of the insulin signaling pathway works as intended by causing a decrease in size. This decrease in size due to insulin signaling downregulation was seen amongst eye discs of the experimental crosses, especially in the InR^{K1409A} eye discs, but also in the $Dp110^{D954A}$ and PTEN eye discs to a lesser extent.

There is another potential venue of research in finding out if amino acid transporter Slimfast is affected by this model. As per Nowak et al. (2013), reduction in the amount Slimfast leads to a decrease in the overgrowth powered by PTEN. Since PTEN is part of the working model for the insulin/hdc signaling pathway, perhaps its interaction with the pathway can be measured after the pathway itself is proven to exist. An important note in this potential endeavor is that Slimfast is found in the digestive areas and the fat body (fat storage of the cell) (Nowak et al., 2013).

There is still a need to ascertain this pathway. Aside from the fact that the pathway linking nutrient restriction to a recently discovered anti-proliferative mTOR pathway regulator is still unknown, this model could be used to help explain certain cancer phenomena. One of these phenomena is cancer cachexia, where the patient wastes away and experiences asthenia, weight loss, anorexia, and anemia. A key part of this disease is that muscle, in addition to fat, is also broken down. This is abnormal compared to a starvation state, where only fat is broken down to generate glucose and ketones (Naveed et al., 2014). One explanation is that downregulated mTORC1 in muscle cells during cachexia prevents the anabolism needed to maintain muscle mass (Duval et al., 2018). This was further proven when Geremia et al. (2022) used mice to demonstrate that activation of Akt-mTORC1 signaling rescued the cachexia-associated muscle wasting. In this situation, the insulin-hdc signaling pathway could provide an explanation. The muscle wastage due to low mTORC1 signaling could be attributed to lower HECA signaling caused by low nutrient levels in the blood. Meanwhile, the tumor responsible for this cachexic state would proliferate without heeding the nutritional state of the body due to broken HECA signaling. Hong et al. (2019) found that microRNA-550a, which affects expression in muscle

systems and is unique to esophageal cancer, is significantly negatively correlated with expression of HECA, which further points to the potential existence of this pathway.

Another area where this model can be applied to starvation based differential chemotherapy. While long-term starvation, dietary, or caloric restriction can especially be detrimental to the health of cancer patients, a short term starvation can give non-cancerous cells protection against chemotherapy. This is because normal cells can respond to nutritional stress, while cancer cells cannot do this. Also, cancer cells tend to rely on glycolysis more for energy metabolism, whereas normally functioning cells mostly rely on oxidative phosphorylation. During periods of starvation, there is increased mitochondrial respiration, which causes increased reactive oxidation species (ROS) production. The increased ROS production is a major life span determinant, and normal cells are able to react in ways that improve their maintenance abilities and resist stress-inducing environmental insults. As a result, short-term starvation before chemotherapy protects normal cells while killing cancer cells more efficiently (Naveed et al., 2014). The proposed insulin signaling model could provide a direct pathway that acts alongside other well-established pathways to explain why cancer cells are unable to react to environmental stresses in the same ways as normal cells. Thus, this model has a lot of pertinence to cancer treatment and must therefore be further confirmed.

Methods

Drosophila Model

The specific fly model used was genetically modified such that the insulin signaling pathway is down regulated via the UAS-Gal4 system and *hdc* clones were generated via the Flippase (Flp)/FRT system. In the UAS-Gal4 system, yeast transcriptional activator Gal4 is expressed only in cells that express a specific cell-lineage gene enhancer. This Gal4 then binds to upstream

activating sequence (UAS), causing the transcription of any protein regulated by UAS. For each fly cross used, a single protein in the insulin signaling pathway had modified expression to the effect of down regulating the pathway. In this model, InR and DP110 signaling was inhibited by expressing the dominant negative mutant forms of both proteins, InR^{K1409A} and $Dp110^{D954A}$. Dominant negative mutants are forms of the mutated protein that have a greater phenotypic expression over and inhibit the function of the WT protein. PTEN was overpressed using the UAS-Gal4 system in a cross.

The Flp/FRT system uses recombinase Flp to cause recombination at flippase recombinase target (FRT) sites. In this model, hsFlp was used, which meant that the cross progeny had to be heat shocked as larvae in order to activate Flp and cause recombination between chromosomal sections containing mutant *hdc* (*hdc*$^{\Delta}$) and green fluorescent protein (GFP). This recombination caused groups of cells expressing either *hdc*$^{\Delta}$ or GFP (WT) clones.

Drosophila Genetics

All the flies had the hsFlp gene in the first chromosome. The second and third chromosomes were fused together and had the desired genetic mechanisms or were a tubby balancer. The desired genetic mechanisms contained FRThdc$^{\Delta}$ and UAS-INR^{8252}, UAS-$DP110^{\Delta 954A}$, or UAS-PTEN for male flies. For the female flies, the desired genes were GFP and GMR-Gal4. The control male flies also expressed FRThdc$^{\Delta}$ as well.

Drosophila Crosses

A ratio of at least five males to ten virgin females were crossed in each vial.

Drosophila Dissections

At the third instar stage, long-bodied larvae were selected and dissected for their heads in *Drosophila* Schneider's Medium. This medium was used so as to make sure the cells in the eye

disc remained well nourished after the death of the larvae. The heads were then fixated in a 4% PFA solution for 15 minutes, washed three times in PBT for 10 minutes each, and then washed in PBTG (blocking solution) for at least an hour. The heads were then fine dissected in PBS for the eye discs. These eye discs were then put on slides.

The eye discs were used for the purposes of this experiment because genetic modifications that affected the eyes did not adversely affect the overall health of the flies.

Heat Shock Protocol

In order to generate the *hdc* mutant clones in the larvae of the crosses, the vials were heat shocked 24-36 hours past the time of egg-laying. The heat-shocking was done by placing the vials in a water bath heated to 37^{o}C for up to two hours. An important note is that the level of the water had to be above the level of the food surface in the vial. This meant that the vials had to be weighed down in order to allow for property heatshocking.

To accomplish the 24-36 heat shock schedule, the breeding flies are allowed to lay eggs for twelve hours. After twelve hours, the flies are transferred to a new vial to lay eggs. Meanwhile, the larvae in the previously occupied vials are allowed to develop for 24 hours before the vial is heat shocked.

Fly Food and Housing

All flies were fed Bloomington *Drosophila* Stock Center's Cornmeal-Molasses-Yeast medium recipe with some small adjustments. The flies were housed at 25^{o}C.

Image Analysis

Leica SP8 confocal microscope was used to take the images in Figure 2.

Table 1

Key Resources Table

Reagent or Source	Resource	Identifier
Chemicals		
Schneider's Drosophila Media	GIBCO	21720024
Experimental Models: Organisms/Strains		
FRThdc$^{\Delta}$	Yu Lab, Ref. Li. et al.	N/A
UAS-InR^{K1409A}	Bloomington Drosophila Stock Center	8252
UAS-DP110$^{\Delta 954A}$	Bloomington Drosophila Stock Center	8251
UAS-PTEN	Gift from Dr. Duojia Pan lab	N/A
GMR-Gal4	Yu Lab, Ref. Li. et al.	N/A

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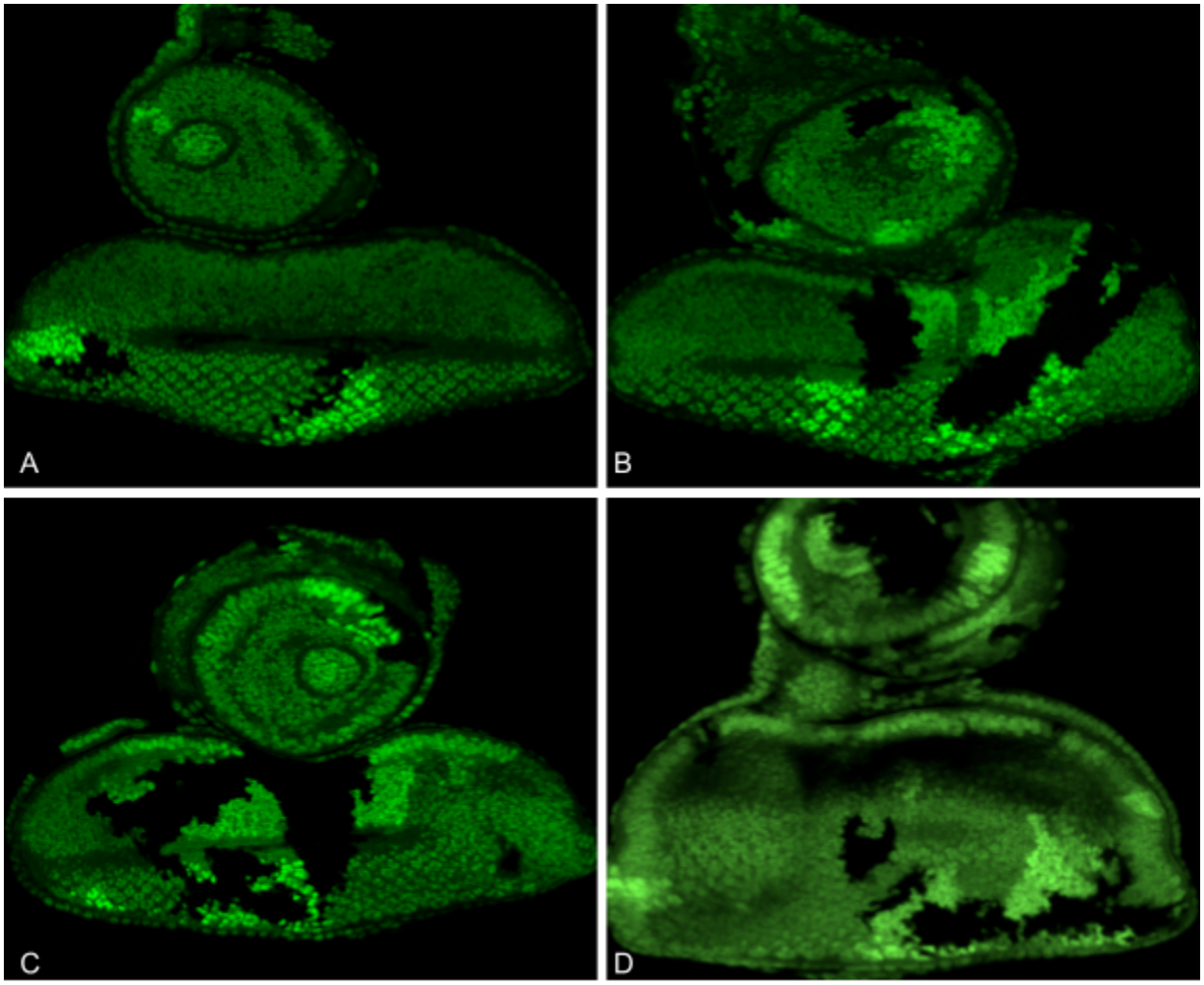
the Pan Lab for providing me with the fly strains. This work was funded by the National Science Foundation (Award #2115690).

Figure 2

Confocal Images of Eye Discs



Note. These are confocal images of the eye discs of larvae with or without insulin signaling down regulation. **2A** is from control larvae with no insulin signaling downregulation. **2B**, **2C**, and **2D** are from larvae with insulin signaling impairment at InR, Dp110, and PTEN respectively.

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