Ingestion of diadzein leads to an increase in ERR-dependent larval lethality in *Drosophila melanogaster*

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Abstract

Diet plays an important role in the cellular pathways responsible for metabolic regulation in living organisms. Previous studies in *Drosophila melanogaster* have shown that ingestion of soy products leads to a decrease in larval survival by preventing larval molting between instar stages. Because these molting stages are hormonally regulated, we hypothesized that this larval death may be due to isoflavones, plant-derived estrogen-like hormones, found in the soy products. When w^{118} *Drosophila* were fed different concentrations of the isoflavone diadzein we saw that there was a significant increase in larval death when compared to the control. Based on its high homology to the estrogen receptor, we hypothesized that this increase in larval death is due to its interaction with the orphan receptor estrogen-related receptor (ERR). When *Drosophila* lacking the ERR were fed similar diadzein concentrations the previously reported larval death was abolished, supporting our hypothesis. This not only establishes diadzein as an ERR ligand, but may also be significant in possible dietary considerations in people suffering from triple negative breast cancer. This especially difficult to treat carcinoma is characterized by loss of typical therapeutic cell receptor targets, such as the estrogen receptor and HER-2, however not only do patients express the ERR, but it is upregulated. A better understanding of the interaction of isoflavones with the ERR could open the door to more effective therapeutics.

Introduction

Diet plays an important role in metabolic pathways, because as a substance is broken down by the body its components are then used throughout the body. These components have many different uses all over the body, and one major place that they have a crucial role is in intracellular signaling. Many of the processes that our bodies undergo are controlled through signaling pathways that are activated using a receptor/ligand mechanism (Bardet *et al*. 2006). Think of this mechanism as a lock and key mechanism, where the receptor is the lock and if the goal is to get the other side of the door, the key (ligand) is needed to fit in the lock and unlock the door. In cases such as this, a receptor many only have one specific ligand that can activate, or in some instances inhibit, the pathway that the receptor controls. Many of these ligands are hormones that we obtain through our diet through or create ourselves. As a result, these hormones play an important role in regulating cellular processes.

In the case of hormones that are regulated through diet, a classic example would be the case insulin and glucagon regulating the amount of glucose present within the blood. When humans consume and break down food the sugar glucose is released; this molecule is vital for survival as it is the major energy source needed for other cellular processes. In times of high glucose levels, such as after a meal, the pancreas produces insulin, a hormone, which signals for some of amount of the glucose that has been ingested to be stored for a later time when glucose is not at such a high amount. When blood glucose levels drop glucagon, another hormone, is released that causes the release of glucose from the liver where it is stored (Aronoff *et al.* 2004). This balancing act between glucagon and insulin must be perfectly maintained or the is the risk of suffering

from diabetic shock or death. In this case the relationship between glucagon and insulin ensures that the energy needs of the body are always met, while at the same time making sure the system is not flooded with so much glucose that metabolic pathways become over-stimulated. To further understand how hormones that are obtained through our diet can influence cellular processes, researchers often use mode organism to mirror these processes.

Model organisms play a crucial role in scientific research, as they help researches understand different biological processes that pertain to humans using a non-human organism. These model organisms have genes/pathways that are homologous to those that are found in humans, and by subjecting these homologous genes/pathways to different circumstances, research can predict the effect these circumstances could have on humans. This is why model organisms are the first subjects for drugs that developers want to one day sell to the human population. There are certain species that are considered to be better model organisms over others, due to differing biology of different organisms and their ease of use. Though this may vary due to the type of study being done, a good model organism is a species that is cheap to keep and reproduce in a laboratory, has a short maturation period from birth to adult, produces many offspring, has a genome that is easy to manipulate, and multiple phenotypes for its features. *Drosophila melanogaster* is an organism that processes these characteristics, ans has been a favored model organism since the beginning of the last century due to large number of developmental processes that have been conserved between flies and vertebrates. Genetic manipulation pertaining to *Drosophila* also very simple as the *Drosophila* genome only contains four

chromosomes. This has made *Drosophila melanogaster* a great tool in understanding biological processes in vertebrates.

One of these conserved biological pathways focuses on the activation of an orphan receptor, called the Estrogen-Related Receptor (ERR). Orphan receptors are receptors found in the body that do not have an identified ligand; a lock without a key. Identifying the ligand for orphan receptors in the body would allow further understanding of the orphan receptor. While the ligand for the ERR is unknown, it has been shown that the ERR interacts with estrogen signaling, a key factor in the promotion of breast cancer (Bardet *et al*. 2006), which causes the death of 7% of the female population every year (Foulkes *et al*. 2010). This receptor is especially interesting as it is upregulated in triple negative breast cancer, which is a form of breast cancer that lacks the three receptors, the estrogen receptor (ER), progesterone receptor (PR), and HER2, most commonly used as targets of breast cancer treatment. Gaining a better understanding of the ERR, such as identifying a ligand for the receptor, could lead to the development of cancer therapies that target the ERR that act in a similar manner to therapies that target the other receptors, like HER2, found on non-triple negative breast cancer.

The HER2 receptor is amplified and overexpressed on 25% to 30% of breast cancers (Slamon *et al.* 2001). This type of breast cancer can be quite aggressive but there has been success in treating it using monoclonal antibodies. Antibodies are molecules that can be produced to specifically bind to only certain proteins on cells. They then signal to the cells of the immune system to come and destroy the cell that it is attached to. These antibodies being specific of HER2 allows them to only bind to the HER2 receptor on cancer cells which, when bound, will signal to the immune system cells to

preferentially bind and lysis the cancer cell that is over-expressing the HER2 receptor (Slamon *et al.* 2001). If the ligand fro ERR was identified it could be used to produce a similar system where binding to the ERR triggers cellular events that cause the death of the cancerous cells.

The ERR has been conserved in *Drosophila melanogaster* and has been shown to be critical to the metabolic transition during development (Tennessen et al. 2011). Twenty-four hours after *Drosophila* eggs are laid, they hatch into first instar larvae, twenty-hour hours later they molt into second instar larvae, after another twenty-four hours they molt into third instar larvae, and twenty-four hours they will have pupated, forming a cocoon-like structure around themselves (Johnston 2002). The molting between these instar larval stages is a carefully controlled process and the expression of the ERR in mid-embryogenesis as an essential regulator of carbohydrate metabolism, making it an extremely important factor within the molting process; so much so, that studies have shown that when the ERR becomes inactivated the larvae die during the second instar stages (Tennessen et al. 2011). This shows that the ERR plays a critical role in the transition through the larval stages, and thus gaining a better understanding of how the ERR is regulated in *Drosophila* larvae could lead to a better understanding of the pathway in humans as well. One possible step in this direction is by identifying a ligand for the ERR.

Previously it has been shown that when *Drosophila* larvae were feed soy products such as tofu and soy sauce there was a decrease in the survival rate during specific larval instars; the ability of the larvae to transition from one instar into the next instar stage was being disrupted. This was very similar to pervious studies where the ERR was

inactivated, causing an inability of the larvae to molt from one instar stage to the next (Tennessen et al. 2011). Due to these similarities in increased larval death when larvae were fed soy products and when the ERR was inactivated, it was hypothesized that a molecule within the soy products were acting as a possible ligand for the ERR and interacting with the receptor in a way therefore inhibiting the molting cycle and causing an increase in larval death.

Soy products contain hormones called isoflavones, which are estrogen-like molecules, and as described earlier, the ERR is known to interact with estrogen signaling. Due to this relationship it has been hypothesized that isoflavones could interact with the ERR. In pervious research performed during my summer 2016 ACRE, I supported this hypothesis by feeding daidzein, an isoflavone found in soy products, to *ERR-LBD-GFP Drosophila melanogaster,* a mutant *Drosophila* stock that has had a fluorescent marker attached to their ER-ligand binding domain that will be activated when something binds to it. Using this system we determined that daidzein was interacting with the ERR, causing the activation of the fluorescent marker, with unfed flies displaying fluorescence in their digestive tract, while *ERR-LBD-GFP* larvae fed daidzein did show fluorescence in their digestive tract.

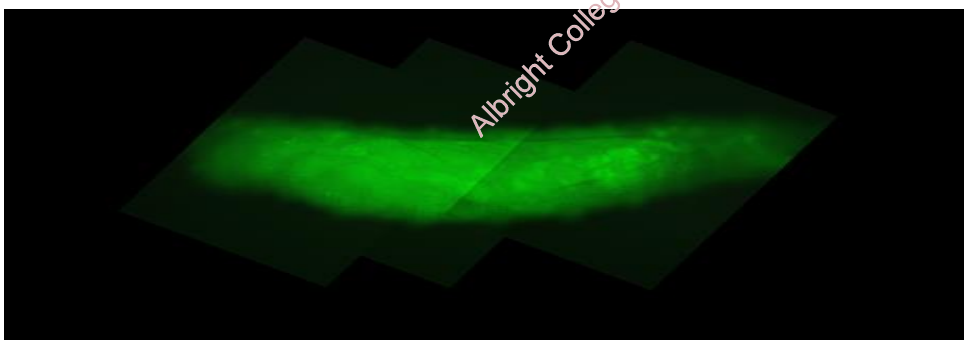

Figure 1. 3rd instar *ERR-LBD-GFP Drosophila melanogaster* larvae when fed 0μM daidzein yeast paste show no fluorescence in their digestive system.

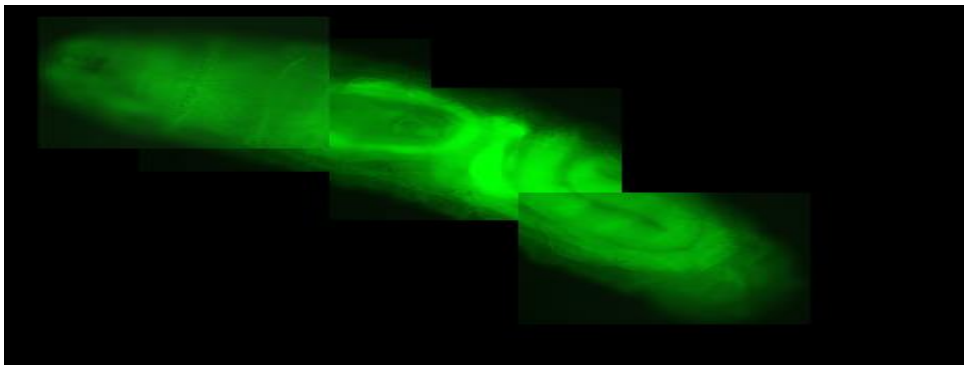

Figure 2. 3rd instar *ERR-LBD-GFP Drosophila melanogaster* larvae when fed 100μM daidzein yeast paste show GFP expression indicating ligand binding.

In this study the effects of daidzein binding to the ERR were further explored. We show that daidzein binding to the ERR causes an increase in larval death in *wildtype*^{118} *Drosophila melanogaster*, larvae between the second and third instar molts when the larvae are fed yeast paste food containing various concentrations of the isoflavone daidzein. To confirm the role of the ERR, ERR knockout larvae *yw;E[EP]ERR Drosophila melanogaster*, were also fed differing concentrations of daidzein but do not show the same increase in their larval death between instar stages. Thus we support that the binding of daidzein to the ERR disrupts the molting pattern of the *Drosophila* during their larval stages and therefore causes an increase in larval death.

Methods

Fly genome types

Two groups of *Drosophila melanogaster* were used during this research, a *w*^{118} *Drosophila melanogaster* (wild type) stock and a *yw; P[EP]ERR Drosophila melanogaster* (ERR knockout mutant) group that were gifted by Alana O'Reilly from Fox Chase Cancer Center, Philadelphia Pa.

Daidzein Yeast Paste

Water and yeast were mixed together to produce yeast paste, and daidzein was mixed into the paste to create yeast paste with concentrations of 200μM and 500μM daidzein.

Grape juice plates

Grape fruit plates make up the bottom of the egg-laying chambers. They are a combination of agar, water, and grape juice, and are stored at 4℃.

Survival Chambers

Four egg-laying chambers were set up, two containing five female and two male w^{118} *Drosophila* each and two with five female and two male yw; P[EP]ERR *Drosophila* each. Each chamber contains a grape juice plate that has 0μM daidzein yeast paste as a food supply for the flies. The chambers were incubated overnight. The next day the adult flies were disposed of and the eggs laid over night were counted. Three survival chambers were set up, one containing 0μM daidzein yeast paste (control), 200μM daidzein yeast paste, and 500μM daidzein yeast paste. Twenty eggs from the w^{118} *Drosophila* were placed into each survival chamber and incubated for twenty-four hours. Three other chambers were set up, one containing 0μM daidzein yeast paste (control), 200μM daidzein yeast paste, and 500μM daidzein yeast paste. Twenty eggs from the ERR mutant *Drosophila* were placed into each survival chamber and incubated for twenty-four hours. The first instar larvae from each plate were counted and transferred to a new survival chamber with concentration matched yeast paste. The chambers were incubated for twenty-four hours and the same transfer process was applied to counting the 2nd instars, 3rd instars, and pupa.

Percent Survival

$$\frac{\text{larval stage of interest}}{\#\ \text{1st instar larave}} \times\ 100\% = \%\ \text{survival}$$

Percent survival was then calculated from each of the larval stages. This process was repeated once a week over a twelve week period.

Results

***w*^{118} *Drosophila melanogaster* larvae have increased death rates following daidzein ingestion**

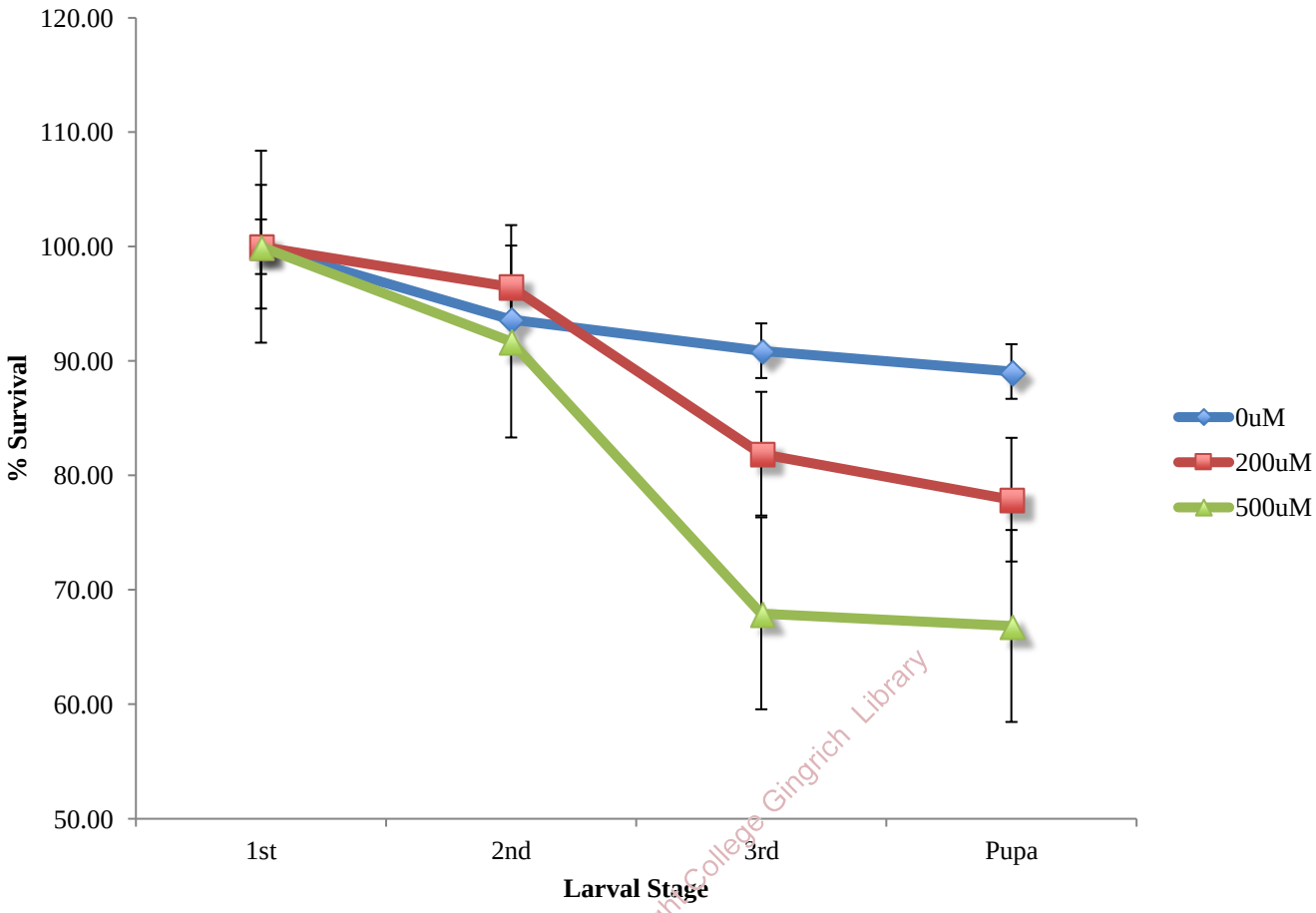

Figure 3. Percent survival of 1st instar, 2nd instar, 3rd instar, and pupa *w*^{118} *Drosophila melanogaster* larval stages when feed 0μM, 200μM, and 500μM daidzein yeast paste. Larvae fed the 0μM daidzein (n=120) yeast paste showed a large percent survival then the 200μM or 500μM daidzein fed larvae. The 500μM daidzein larvae (n=240) had the largest decrease in percent survival, while the 200μM daidzein fed larvae (n=240) had a percent survival the fell in the middle of the two other groups.

The *Drosophila melanogaster* larvae that were fed with the 0μM daidzein yeast paste (control) displayed an 89.09% larval survival from 1st instar larvae to pupa compared to the 77.89% larval survival of the 200μM daidzein yeast paste group and the 66.85% larval survival of the 500μM daidzein yeast paste group (Figure 3). The 500μM daidzein yeast paste group showed a large decrease in larval survival between the 2nd instar larval stage (91.71% survival) and 3rd instar larval stage (67.95% survival). This drop was not seen the 0μM group, while the 200μM group did experience a similar drop in larval survival between the 2nd (96.48% survival) and the 3rd (81.91% survival) instar stages; as well as an additional drop between the 3rd (81.91% survival) instar and pupa (77.89% survival) stages.

ERR Mutant *Drosophila melanogaster* larvae did not demonstrate the same daidzein dependent lethality as *w*^{118}

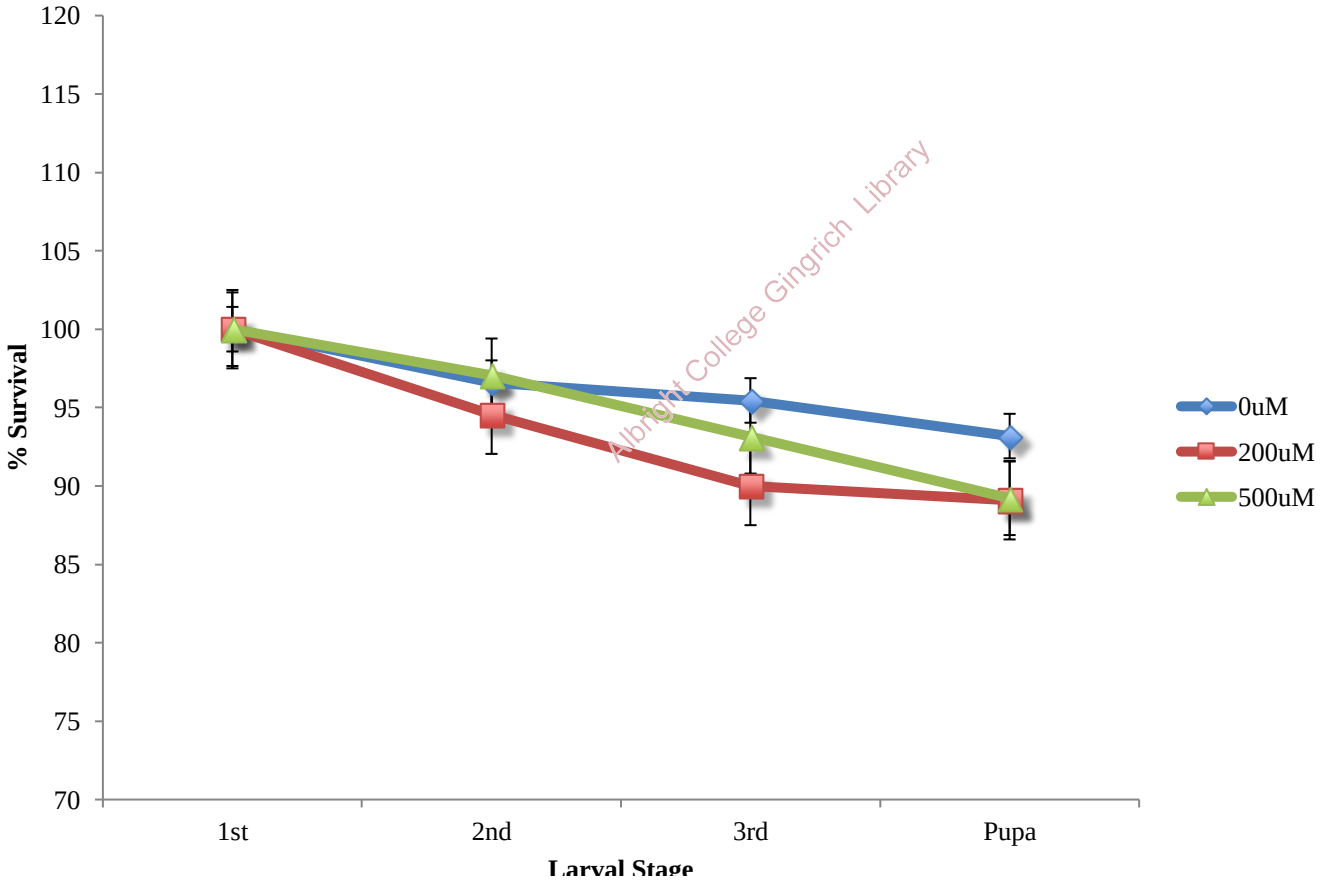

Figure 4. Percent survival of 1st instar, 2nd instar, 3rd instar, and pupa of ERR mutant *Drosophila melanogaster* larval stages when fed 0 μM, 200 μM, and 500 μM daidzein yeast paste. Larvae fed the 0μM daidzein (n=150) yeast paste showed a large percent survival while the 500 μM larvae(n=150) had the smallest percent error and the 200 μM larvae (n=150) fell in the middle. The difference between the percent survival of each group was relatively small, with the final percent survival of the 200 μM and 500 μM larvae being almost identical.

The ERR mutant *Drosophila* larvae fed 0μM daidzein yeast paste (control) had a percent survival of 93.18% from the first instar larvae to the pupal stage (Figure 4). The 200μM and 500μM daidzein yeast paste groups also experienced small decreases in their larval survival. The 200μM group experienced a drop between the 1st and 2nd instar stages to 94.54% survival, the 2nd and 3rd instar stages 90.00% survival, and the 3rd instar and pupa stages to 89.09% survival. The 500μM group had drop from 97.06% survival in the second instar stage to 93.14% survival in the instar, and then another small decrease in survival to 89.21% survival in the pupal stage.

Discussion

We demonstrated that feeding *Drosophila melanogaster* larvae increasing concentrations of daidzein, a hormone found in soy products, correlates with a decrease in larval survival. The w^{118} *Drosophila* larvae that were not fed daidzein had a much higher rate of larval survival when compared to the larvae that were fed 200μM and 500μM daidzein concentrations, especially during the 2nd to 3rd instar transition. This is further supported when comparing this data with the larval survival of the ERR mutants. While the ERR mutant larvae did experience a decrease in larval survival when fed increasing concentrations of daidzein, it was not to the level of larval death seen in the w^{118} *Drosophila* larvae. This data supports that the interaction of the daidzein with the

ERR leads to the disruption of the molting behavior of the *Drosophila* larvae leading to larval death.

These findings further underlie the importance of diet for certain individuals. In the case of the ERR, these results could be relevant to individuals suffering from triple-negative breast cancer. Triple-negative breast cancer (TNBC) is characterized by tumors that lack the expression of three receptors, estrogen receptor (ER), progesterone receptor (PR), and HER2, that are commonly targeted by breast cancer therapies (Foulkes *et al*. 2010). TNBC is very difficult to treat and very deadly; in 2007 78.6% of women who were diagnosed with triple negative breast cancer were alive after five years compared to 97% women diagnosed with non-TNBC. While TNBC has been shown to lack all three common receptor normally used to treat breast cancer, it has been shown that ERR is upregulated in this type of breast cancer (Foulkes *et al*. 2010). Therefore a better understanding of the mechanisms behind the interaction of the ERR and isoflavones like daidzein could bring insight into ways to treat triple negative breast cancer. This study supports that daidzein is acting as a ligand for the ERR in such a way to cause a phenotypic change with the organism, which may be similar to humans.

My past research only supports that the daidzein is binding to the ERR and this study shows that the binding causes significant changes within *Drosophila melanogaster*, but both of these past studies do not explan what the binding of the ligand does to the ERR in humans. For example, it had been shown that increased expression of ERR has lead to an increase in cell proliferation, a characteristic of cancer, but does the binding of the ligand, daidzein, to the ERR increase the expression of ERR or inhibit it? Therefore,

a new, more complete mechanism needs to be established as daidzein binding may inhibit of activate ERR function leading to a wide variety of consequences.

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