Rice University

Broad-Complex, A Mediator of Ecdysone Induced Stress Response in Drosophila melanogaster

by

Veronica Hall-O’Connell

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE

Doctor of Philosophy

Approved Thesis Committee:

Dr. Michael C. Gustin, Associate Professor, Chair, Biochemistry and Cell Biology

Dr. George N. Bennett, Professor, Biochemistry and Cell Biology

Dr. Kathleen M. Beckingham, Professor, Biochemistry and Cell Biology

Dr. Mary Ellen Lane, Assistant Professor, Biochemistry and Cell Biology

Dr. Lisa M. Meffert, Assistant Professor, Ecology and Evolutionary Biology

Houston, Texas

June 2004
INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform 3170792

Copyright 2005 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346
June 2004

Abstract

Broad-Complex, a mediator of ecdysone induced stress response in Drosophila melanogaster

By

Veronica Hall-O’Connell

The better an animal can react to stressful conditions the longer it can live and possibly reproduce. Stress response and lifespan are under the control of two pathways of the endocrine system: the insulin/IGF-1 signaling pathway and a steroid hormone-signaling pathway. Mutations that alter the expression or function of the components of these pathways increase stress tolerance and lifespan. In D. melanogaster, mutants that reduce signaling the steroid hormone ecdysone show an increase in stress tolerance and lifespan. Although ecdysone signaling is important for stress resistance and lifespan, the molecular mechanism responsible remains to be elucidated. My thesis research focused on the ecdysone-induced family of zinc finger transcription factors known as the Broad-Complex (BR-C) as a possible downstream target of ecdysone mediated stress resistance and lifespan. I examined the role of BR-C in stress resistance and longevity by measuring the survival under varying conditions of flies that have altered BR-C expression. BR-C expression was altered by one of three methods: a P-element insertion into the BR-C loci that causes the misexpression of BR-C transcripts, heatshock overexpression of BR-C transgenes, and reducing BR-C transcript levels by RNAi. The results from these experiments suggest a complex correlation between BR-C expression and stress response.
Global overexpression or underexpression of BR-C suggests a positive correlation between BR-C expression levels and stress resistance. Misexpression of BR-C transcripts by P-element insertion into the BR-C loci indicates an inverse correlation between BR-C expression levels and stress resistance. My thesis research allows us to conclude that BR-C mediates stress resistance and may be the downstream target of ecdysone mediated stress resistance at the molecular level.
Acknowledgements

I would like to thank my advisor, Dr. Michael Gustin, for his ingenious ideas and advice. His motivation and enthusiasm throughout this project encouraged me to move forward even when times seemed bleak.

To my thesis committee, Drs. Bennett, Beckingham, and Lane, I owe a debt of gratitude. Their encouragement throughout the years was more than I could have expected.

I would like to thank Dr. Stern and for his guidance and teachings of fly genetics. He took time out of his busy schedule to discuss fly crosses and markers for each cross although I was not in his lab. His mentoring helped me make it through my graduate school career.

I am truly grateful to my friends who have stood by me during the good times and the dark times. For always making me laugh when I was down and pointing out how things could be worse, like “so what if your results are bad, you could have Ebola.” I would especially like to thank my valued friend, Jaime Becnel, who is well versed in the art of bovine feces. Jaime would sit for hours with me discussing my results and how to present those results. She can truly spin hay into gold. Her influence and help went beyond what anyone could expect from a friend. I would also, like to thank Liz McCormack for helping me organize my thoughts and write this thesis. Her editing skills are amazing. She can reorganize any sentence into a strong statement. Her ability to aid others is unbelievable.

Last, but certainly not least, I would like to thank my family for their love and support. I would like to thank my mother for always encouraging me and reminding me
how wonderful I am. She can always say the right thing when I need it. I would like to thank my step-dad, Richard, for his wonderful advice: “Just get drunk”. I would like to thank my brother for always introducing me to his friends as “this is my sister Veronica, she is a professional student.” No, I am not a professional student. I am finally done.
# Table of Contents

Chapter 1 ........................................................................................................... 1  
1.1 Introduction............................................................................................... 1  
1.1.1 Stress and Longevity in *Caenorhabditis elegans* .................................. 2  
1.1.2 Stress, Reproduction, and Aging *Drosophila melanogaster* ................. 4  
1.2 Broad Complex: ....................................................................................... 8  
1.2.1 The Role of BR-C in Metamorphosis .................................................... 16  
1.2.2 The Role of BR-C in Oogenesis ............................................................ 21  
1.3 Hypothesis............................................................................................... 22  

Chapter 2: Materials and Methods .................................................................. 24  
2.1 Fly Cultures............................................................................................... 24  
2.2 Fly lines.................................................................................................. 24  
2.2.1 c754 .................................................................................................. 24  
2.2.2 HS BR-C isoforms .............................................................................. 27  
2.2.3 Construction of UAS-BR-C lines ......................................................... 29  
2.2.4 Construction of BR-C RNAi Lines ..................................................... 34  
2.2.5 Construction of the Tet-O Gal4 Driver Line ....................................... 35  
2.2.6 Expression of the RNAi Constructs with Either Act-5 Gal4 or Hs-Gal4 .... 35  
2.3 Phenotype Assays ................................................................................... 36  
2.3.1 Starvation Assays .............................................................................. 36  
2.3.5 Lifespan Assay in Demography Cages .............................................. 37  
2.4 Molecular Assay .................................................................................... 39  
2.4.1 Plasmid Rescue of c754 .................................................................... 39  
2.4.2 Reverse Transcription PCR (RT-PCR) ............................................ 39  
2.4.3 Quantitative Real Time RT-PCR (Q-PCR) ....................................... 40  

Chapter 3: The mutant c754 ......................................................................... 42  
3.1 Background on c754 mutant .................................................................. 42  
3.2 Determining the Gene Disrupted by the c754 P-element Insertion .......... 43  
3.2 Determining the Gene Disrupted by the c754 P-element Insertion .......... 44  
3.3 Stress and Lifespan phenotype in c754 ................................................... 44  
3.5 Discussion of Results ............................................................................ 70  

Chapter 4: Lifespan Increase by BR-C Overexpression is Isoform Dependent .... 74  
4.1 Generating Overexpression Transgenic lines ......................................... 74  
4.2 Overexpression of BRC-Z1 Increases Starvation Tolerance .................... 75  
4.3 Overexpression of BRC-Z2 Increases Starvation Tolerance .................... 77  
4.4 Overexpression of HS-BRC-Z3 Does Not Change Starvation Tolerance .... 81  
4.5 Overexpression of HS-BRC-Z4 Increases Starvation Tolerance ............... 81  
4.6 Discussion ............................................................................................. 86  

Chapter 5: Underexpression of BR-C isoforms using RNAi .......................... 90  
5.1 Background on RNAi ............................................................................. 90  
5.2 Driving Expression of the RNAi Construct Using Act-5 Gal4 ................. 93  
5.2.1 Act-5 Gal4 RNAi Directed Towards the Core Domain Results in Pupal Arrest ................................................................. 94
5.2.2 Act-5 Gal4 RNAi Directed Towards the BRC-Z1 Isoform Blocks Development at the Pharate Adult Stage ................................................................. 94
5.2.3 Act-5 Gal4 RNAi Directed Towards the BRC-Z2 Isoform Results in Pupal Arrest ................................................................. 96
5.2.3 Act-5 Gal4 RNAi Directed Towards the BRC-Z2 Isoform Results in Pupal Arrest ................................................................. 97
  5.2.4 Act-5 Gal4 RNAi Directed Against the BRC-Z3 Isoform Requires Two Copies of The RNAi BRC-Z3 Transgene to Observe a Pupal Arrest Phenotype ................................................................. 97
  5.2.5 Act-5 Gal4 RNAi Directed Towards the BRC-Z4 Isoform Results in Partially Eclosed Adults .................................................................................. 99
5.3 Driving RNAi expression with and inducible Gal4 expression ...................... 102
  5.3.1 Flies expressing RNAi BRC-Core Did Not Have a Change in Starvation Resistance ................................................................. 102
  5.3.2 Females Expressing RNAi BRC-Z1 Have a Significant Decrease in Starvation Lifespan ................................................................. 103
  5.3.3 Expression of RNAi BRC-Z2 Did Not Alter Starvation Resistance ........... 108
  5.3.4 Expression of RNAi BRC-Z4 Decrease Starvation Resistance in Females .. 109
  5.3.4 Expression of RNAi BRC-Z4 Decrease Starvation Resistance in Females .. 110
  5.3.4 Expression of RNAi BRC-Z4 Decrease Starvation Resistance in Females .. 111
5.4 Discussion ......................................................................................... 114
Chapter 6: Discussion and Future Work .......................................................... 118
  6.1 Discussion ......................................................................................... 118
  6.2 Future Work .................................................................................... 123
References: .......................................................................................... 127
Figures and Tables

Figure 1.1 The *C. elegans* gonad regulates lifespan by separate signals from the somatic and germline portions of the gonad.........................................................3

Figure 1.2 The *Broad Complex* and transcripts.............................................10
Table 1.1 Alleles of *BR-C*.............................................................................15

Table 2.1 Ingredients for Caloric restriction food...........................................25

Figure 2.1 c754 excision cross............................................................................26

Table 2.2 Primers used......................................................................................28

Figure 2.2 Determining chromosomal location of transgene insertion in transgenic males.................................................................31

Figure 2.3 Determining chromosomal location of transgene insertion in transgenic females...............................................................33

Figure 3.1 c754 is more resistant to osmostress...............................................43

Figure 3.2 Schematic drawing of c754 insertion in the *BR-C*........................45

Figure 3.3 c754 females have an increase in starvation survival compared to the isogenic control females....................................................47

Figure 3.4 c754 males have increased starvation survival compared to the isogenic control males..............................................................48

Figure 3.5 c754 females live significantly longer than the isogenic control females when fed cornmeal agar media...........................................50

Figure 3.6 c754 males live significantly longer than the isogenic control males when fed cornmeal agar food.................................................51

Figure 3.7 c754 females do not live significantly longer than the isogenic control females when fed 0.65X sucrose yeast media..........................53

Figure 3.8 c754 males do not live significantly longer than the isogenic control males when fed 0.65X sucrose yeast media..............................54

Figure 3.9 The lifespan of c754 females decrease compared to the isogenic control females when fed 1X sucrose yeast media.........................55
Figure 3.10 c754 males do not live significantly longer than the isogenic control males when fed 1X sucrose yeast media.................................................................56
Figure 3.11 c754 females have a significant decrease in lifespan compared to the isogenic control females when fed 1.5X sucrose yeast media..................................................57
Figure 3.12 c754 males have a significant decrease in lifespan compared to the isogenic control males when fed 1.5X sucrose yeast media..................................................58
Figure 3.13 Q-PCR amplification plot of BRC-Z1 expression in c754-m and isogenic control females.................................................................63
Figure 3.14 Relative expression of BRC-Z1 in c754-m females compared to isogenic control females based on analysis of Q-PCR results..................................................64
Figure 3.15 Q-PCR amplification plot of BRC-Z2 expression in c754-m and isogenic control females.................................................................65
Figure 3.17 Relative expression of BRC-Z2 in c754-m females compared to isogenic control females based on analysis of Q-PCR results..................................................66
Figure 3.18 Q-PCR amplification plot of BRC-Z3 expression in c754-m and isogenic control females.................................................................67
Figure 3.19 Relative expression of BRC-Z3 in c754-m females compared to isogenic control females.................................................................68
Figure 3.20 Q-PCR amplification plot of BRC-Z4 expression in c754-m and isogenic control females.................................................................69
Figure 3.21 Relative expression of BRC-Z4 in c754-m females compared to isogenic control females based on analysis of Q-PCR results..................................................70
Table 3.1 Summary of c754 lifespan compared to control lifespan under different dietary conditions........................................................................................................72
Figure 4.1 The increased starvation survival by overexpression of BRC-Z1 is dominant in females.................................................................76
Figure 4.2 The increased starvation survival by overexpression of BRC-Z1 is dosage dependent in males .................................................................78
Figure 4.3 The increased starvation survival by overexpression of BRC-Z2 is dosage dependent in females .................................................................79
Figure 4.4 The increased starvation survival by overexpression of BRC-Z2 is dosage dependent in males.................................................................80

Figure 4.5 Overexpression of BRC-Z3 does not alter starvation tolerance in females.....82

Figure 4.6 Overexpression of BRC-Z3 does not alter starvation tolerance in males......83

Figure 4.7 The increased starvation survival by overexpression of BRC-Z4 is dosage dependent in females .................................................................84

Figure 4.8 Overexpression of BRC-Z4 has a dosage effect on increasing starvation tolerance in males.................................................................85

Table 4.1 Summary of mean lifespan under starvation of Hs-BRC transgenic lines compared to controls.................................................................87

Figure 5.1 Overview of RNAi-mediated mRNA degradation ........................................91

Figure 5.2 Act-5 Gal4 expression of RNAi targeting the BRC-core domain results in pupal arrest................................................................................95

Figure 5.3 Act-5 Gal4 expression of RNAi targeting BRC-Z1 results in pharate adults.........................................................................................96

Figure 5.4 Act-5 Gal4 expression of RNAi targeting BRC-Z2 results in pupal arrest...98

Figure 5.5 Act-5 Gal4 expression of RNAi targeting BRC-Z3 does not decrease BRC-Z3 mRNA levels.................................................................100

Figure 5.6 Act-5 Gal4 expression of RNAi targeting BRC-Z4 results in partially eclosed adults..................................................................................101

Figure 5.7 Females expressing RNAi directed towards the core domain do not have a change in starvation resistance compared to females not expressing core RNAi.....104

Figure 5.8 Males expressing RNAi directed towards the core domain do not have a change in starvation resistance compared to males not expressing core RNAi........105

Figure 5.9 Females expressing RNAi directed towards BRC-Z1 have significant change in starvation resistance compared to females not expressing BRC-Z1 RNAi........106

Figure 5.10 Males expressing RNAi directed towards BRC-Z1 do not have a significant change in starvation resistance compared to males not expressing BRC-Z1 RNAi.....107
Figure 5.11 Females expressing RNAi directed towards BRC-Z2 do not have a significant decrease in starvation resistance compared to females not expressing BRC-Z2 RNAi.................................................................109

Figure 5.12 Males expressing RNAi directed towards BRC-Z2 do not have a significant change in starvation resistance compared to males not expressing BRC-Z2 RNAi........110

Figure 5.13 Females expressing RNAi directed towards BRC-Z4 have a decrease in starvation resistance compared to females not expressing BRC-Z4 RNAi..............112

Figure 5.14 Males expressing RNAi directed towards BRC-Z4 do not have a change in starvation resistance compared to males not expressing BRC-Z4 RNAi.............113

Table 5.1 Summary of lifespan under starvation flies expressing BRC-RNAi........115
Chapter 1

1.1 Introduction

The longevity of an organism is linked to the ability of an organism to respond to stress and the loss of resources used in reproduction. Typically, the better an organism can handle stress the longer it lives. Organisms are less likely to reproduce during times of environmental stress; instead, organisms allocate resources to stress survival because progeny would be less likely to survive. In direct contrast to stress resistance, reproduction typically decreases lifespan. This decrease in lifespan with increased reproduction may be evolutionarily favorable because parents would consume fewer resources such as food and water (Sgro and Partridge, 1999).

Aging can be considered a progressive deterioration of the organism that eventually leads to death (Rose and Long, 2002). Aging is not just a random process but is under tight cellular control (Helfand and Rogina, 2003); therefore, links between stress and reproductive signaling pathways and aging signaling or gene targets would provide a molecular mechanism for this physiological observation. Some molecular pathways that regulate aging are under control of the endocrine system.

Two pathways mediate endocrine control of aging: the insulin/IGF-1 pathway and a steroid-signaling pathway (Guarente and Kenyon, 2000; Kenyon, 2001; Tatar et al., 2003). Genetic analysis has shown that both an insulin-like receptor and a steroid receptor mediate lifespan and stress response in both Caenorhabditis elegans and Drosophila melanogaster. The molecular mechanism linking the insulin/IGF-1 and steroid signaling in stress response, aging, and reproduction is beginning to be
understood. However, both the upstream and downstream components of each pathway that allow the insulin/IGF-1 and steroid signaling to regulate stress responses are unknown. In order to fully understand the process of aging, pathway components that mediate insulin and steroid signaling in response to age needs to be identified.

1.1.1 Stress and Longevity in *Caenorhabditis elegans*

A good model for molecular pathways involved in aging has been developed for the nematode, *C. elegans*. The strategy of *C. elegans* to combat stress during larval development is to form a dauer, a state in which the larva is less active, more stress resistant, longer lived, and reproductively inactive (Riddle, 1988). Dauer formation is under the control of DAuer Formation (DAF) genes. Mutations of these DAF genes either block the formation of the dauer or cause the formation of a perpetual dauer (Riddle et al., 1981). It has been demonstrated that certain mutations in the DAF genes cause increased longevity and improve stress response in adult *C. elegans* without inducing dauer formation. These long-lived DAF mutants have alterations in either insulin signaling or steroid hormone signaling (Guarente and Kenyon, 2000; Kenyon, 2001; Tatar et al., 2003).

Regulation of the insulin and steroid signals that alter lifespan is partly mediated by the gonad (Arantes-Oliveira et al., 2002; Hsin and Kenyon, 1999; Libina et al., 2003; Riddle, 1999). The origin of the insulin regulated longevity signal coming from the gonad is the somatic whereas the origin of the steroid mediated longevity signal is the germline cells (Arantes-Oliveira et al., 2002; Hsin and Kenyon, 1999) (Fig. 1.1). Ablation of the entire gonad (both germline and somatic cells) does not increase lifespan indicating that the increase in lifespan seen when the germline is removed is not due to
Figure 1.1 The *C. elegans* gonad regulates lifespan by separate signals from the somatic and germline portions of the gonad. InsR = DAF-2 insulin receptor, Fkh = DAF-16 forkhead transcription factor. SterR = DAF-12 orphan nuclear receptor. From Hsin and Kenyon (1999)
loss of reproductive capabilities. In regards to controlling longevity, the germline and somatic cells give off opposing signals. The insulin/IGF-1 signaling and steroid signaling pathways mediate signals from the gonad for longevity and stress response.

The insulin/IGF-1 signaling pathway and the steroid hormone-signaling pathway determine the lifespan and reproduction of *C. elegans*. The insulin receptor homolog, Daf-2, transmits a signal to Age-1, a phosphatidylinositol-3-OH kinase, to inhibit either directly or indirectly the translocation of Daf-16, a forkhead transcription factor, to the nucleus (Apfeld and Kenyon, 1998; Dillin et al., 2002; Friedman and Johnson, 1987; Henderson and Johnson, 2001; Kimura et al., 1997; Larsen et al., 1995; Lee et al., 2001; Lin et al., 1997; Lithgow et al., 1995; Morris et al., 1996; Murphy et al., 2003; Ogg et al., 1997; Tissenbaum and Ruvkun, 1998; Wolkow et al., 2000). Nuclear Daf-16 is required for the transcription of various stress response genes (Murphy et al., 2003). In addition to the Daf-2 insulin-signaling pathway, lifespan in *C. elegans* is also altered by mutations in the nuclear hormone receptor Daf-12, a homolog to the steroid hormone receptor, EcR, in *Drosophila* (Antebi et al., 2000; Hsin and Kenyon, 1999). Daf-12, like Daf-16, mediates signals from the germline that alter lifespan of the animal (Hsin and Kenyon, 1999).

1.1.2 Stress, Reproduction, and Aging *Drosophila melanogaster*

Like *C. elegans*, the lifespan of *D. melanogaster* is also dependent on insulin signaling and steroid signaling. *D. melanogaster* is an ideal model organism for genetic studies because it has a relatively short lifespan, easy maintenance, powerful genetic tools, and a fully sequenced genome. In addition to the powerful genetic tools of *D. melanogaster*, it is a model organism for studying aging because flies are capable of
reproduction shortly after eclosion and most cells in the fly are post-mitotic (Helfand and Rogina, 2003).

Through the use of caloric restriction experiments and several mutants, the knowledge of the molecular pathways involved in aging in *Drosophila* has been enhanced. Caloric restriction is a 30-40% decrease in calories consumed by the animal, which affects longevity and reproduction of the animal (Britton et al., 2002; Good and Tatar, 2001).

In *D. melanogaster*, caloric restriction is simulated by lowering the nutrient quality of the food, but not the quantity of food available for consumption. Altering the yeast content, a major contributor of nutrients, of the food causes changes in both reproduction and lifespan (Good and Tatar, 2001). If no yeast is present in the food, there is an increase in death and a decrease in reproduction suggesting that yeast is necessary for both lifespan and reproduction (Good and Tatar, 2001). At low levels of yeast, there is an increase in lifespan and a decrease in reproduction: the opposite is seen with high levels of yeast (Chapman and Partridge, 1996; Good and Tatar, 2001; Pletcher et al., 2002). Insulin signaling coordinates cell metabolism with calorie intake suggesting that altered lifespan with caloric restriction is mediated by insulin signaling or that insulin signaling regulates the caloric-dependent set point (Britton et al., 2002).

The insulin-like receptor substrate, *chico*, increases lifespan and decreases reproduction when mutated (Clancy et al., 2001). Homozygous female *chico* mutants have a 48% increase in mean lifespan compared to controls, but homozygous male *chico* mutants have a decrease in mean lifespan. Heterozygous *chico* mutants have an increase in mean lifespan in females and males of 36% and 13% respectively. In addition, the
heterozygous mutant females have a decrease in fertility and the homozygous female is infertile. It has been show that a decrease in female fertility resulted in an increase in lifespan (Sgro and Partridge, 1999). The female sterile mutant, \( ovo^{DL} \), has an increase in lifespan compared to wild type females (Clancy et al., 2002). One might argue that the increased lifespan of \( chico \) mutants is the result of trade-offs between reduced reproduction and longevity. The increased lifespan determined for \( chico \) is not completely due to a decrease in reproduction because the mutants of \( chico \) live significantly longer than sterile \( ovo^{DL} \) females. The maximum lifespan of \( chico \) mutants is seen when they are fed standard food, while the wild type live longer when calorie restricted (Clancy et al., 2002). Under caloric restriction conditions, \( chico \) mutants have a decrease in lifespan (Clancy et al., 2002). Therefore, \( chico \) mutants behave as if the caloric restriction set point has changed (Clancy et al., 2002).

\( chico \) encodes the protein substrate for the \textit{insulin-like receptor} (\( InR \)), a daf-2 homolog that also plays a role in lifespan determination (Bohni et al., 1999; Tatar et al., 2001). Mutations in \( InR \) cause an 85% increase in mean lifespan (Tatar et al., 2001). Ovaries of \( InR \) mutants morphologically resemble the ovaries of wild type flies in diapause. Diapause is a state in which reproduction halts until environmental conditions are more favorable for progeny survival. Homozygous \( InR \) mutants are sterile. It is unclear if the increase in lifespan of \( InR \) mutants is caused solely by loss of reproductive capabilities or a combination of reproductive loss and defective insulin signaling. Both the \( chico \) and \( InR \) mutants support the idea that aging is under control of the endocrine system by insulin signaling.
Insulin signaling is not the only pathway that affects stress and lifespan, but as seen in *C. elegans*, steroid signaling decreases lifespan and stress tolerance. The *ecdysone receptor* (*EcR*) is a nuclear hormone receptor protein that is activated by the steroid hormone ecdysone. Homozygous mutants of the EcR are lethal; however, heterozygous mutants are stress resistant and live longer than their controls (Simon et al., 2003). Similar effects of increased lifespan and stress resistance are seen in both ligand binding and DNA binding mutants of *EcR* (Simon et al., 2003). Specifically, a ligand binding domain mutant of the *EcR* leads to 40% and 50% increases in lifespan in males and females respectively (Simon et al., 2003). Consistent with these results, interfering with the biosynthetic pathway of ecdysone causes a change in stress resistance. *DTS-3* is a temperature sensitive mutant in the ecdysone biosynthetic pathway. At elevated temperatures, *DTS-3* females have a 42% increase in lifespan with no change in lifespan for *DTS-3* males (Simon et al., 2003). In addition to mutants in insulin and steroid signaling in longevity and stress tolerance, there are other long-lived mutants that alter aging and stress response with no known link to insulin and steroid signaling.

Two long-lived mutants are *methuselah* and *I’m not dead yet* (*mth* and *indy*) (Lin et al., 1998; Rogina et al., 2000). Mutations in *mth*, which encodes a g-protein coupled receptor, increases mean lifespan by 35% and increases survival to starvation, heat and oxidative stress (Lin et al., 1998). Mutations in *indy* double the mean lifespan (Rogina et al., 2000). *indy* encodes a sodium dicarboxylate cotransporter; a membrane protein that transports Krebs cycle intermediates (Rogina et al., 2000). The fact that there is no current evidence that *mth* or *indy* participate in insulin or steroid signaling does not exclude *mth* or *indy* from being components of either insulin or steroid signaling.
pathways. These long-lived mutants are a good start to begin understanding the molecular mechanism by which aging is controlled I have recently uncovered an additional long-lived stress resistant mutant that may be a possible downstream target to ecdysone induced stress resistance and lifespan.

This long-lived stress resistant mutant is a P-element insertion in the intronic region separating the first exon of those transcripts derived from the P167 promoter and the exon containing the translation start site of the Broad-Complex (BR-C) in D. melanogaster. Ecdysone, a steroid hormone, induces BR-C expression suggesting a possible role for the BR-C in steroid-signaling stress response.

1.2 Broad Complex:

The life cycle of Drosophila can be divided into several morphological distinct stages: embryogenesis, three larval stages, a pupal stage, and adult stage. Embryogenesis lasts approximately 24 hours after which first instar larvae emerge. There are two additional larval stages, known as second and third instar. The first and second instar stages last approximately 24 hours. The third instar larval stage lasts approximately two days in which the larvae enters metamorphosis and transforms into a pupa. The third instar larval stage can be divided into three time points: early (72-86 hours after laying), mid (86-114 hours after laying) and, late (112-120 hours after laying). A 15-hour period between the third instar stage and the pupal stage is known as the prepupal phase. Four to five days after entering the pupal stage, adult flies eclose. The transition from second to third instar larvae and prepupae to pupae is marked with an increase in the steroid hormone, ecdysone, levels (Buszczak and Segraves, 2000).
The increase in ecdysone levels during these transitions triggers the transcription of genes known as early puff genes, which then regulate the transcription of late puff genes (Ashburner et al., 1974). Ecdysone regulated genes fall into two classes: class 1 and class 2 (Karim and Thummel, 1992). Class 1 genes are induced by low levels of ecdysone and repressed by high levels of ecdysone. Class 2 genes are induced by high levels of ecdysone and the timing of gene expression is unaffected by increasing levels of ecdysone. *BR-C* has transcripts that can be classified as either class 1 or class 2 ecdysone regulated genes (Karim and Thummel, 1992).

The *BR-C* spans a 100 kb region on the X-chromosome at position 2B5 (Fig 1.2) (Belyaeva et al., 1980, Kiss et al., 1988). It consists of 14 exons, 4 promoters, and 4 polyadenylation sites (Fig. 1.2). The *BR-C* locus was initially defined by mutations that fall into one of three groups of complementing alleles: br (broad), rbp (reduced bristle on palpus) and 2bc (Kiss et al., 1988). A fourth group of alleles which do not complement any of the three above alleles are known as npr (non-pupariating) and are considered null mutations (Kiss et al., 1988). A major role of the br*+* allele in morphogenesis is elongation and eversion of the appendages and the tanning and hardening of the cuticle. rbp*+* is responsible for the development of muscles and bristles. In addition to being responsible for muscles and bristles, rbp*+* along with 2bc*+* plays a role in formation of the gut. Fusion of discs to form a continuous adult epidermis is also mediated by 2bc*+* (Kiss et al., 1988).

The *BR-C* encodes six protein isoforms containing one of four pairs of C2H2 zinc finger domains: BRC-Z1, BRC-Z2, BRC-Z3 and BRC-Z4 (Fig 1.2). All *BR-C* transcripts share a common amino terminus known as the core region (Bayer et al., 1996; DiBello et
Figure 1.2 The Broad Complex and transcripts A) The molecular organization of the BR-C gene. The BR-C spans a 100 kb region in the X-chromosome with four promoters and fourteen exons. B) The BR-C gene expresses six transcripts all possessing the same N-terminus BTB domain (core region in red). The C-terminus in the six different transcripts differ by the four different zinc fingers encoded: Z1 (blue box), Z2 (light blue box), Z3 (pink box) and Z4 (yellow box). The Z1 zinc finger is in three transcripts differing from each other by the linker region (purple) between the core and the zinc finger. Both Z1 and Z2 are transcribed from both the P_{120} and P_{165} promoters, this shown as two possible promoters in their transcripts in B. Z3 and Z4 are transcribed from the P_{165} promoter. Green boxes are initial exons containing the promoters, orange boxes exons encoding untranslated regions, and red boxes are the core region. Modified from Tzolovsky et al. 1999.
al., 1991). The core region is a 114 amino acid stretch known as a BTB (Broad-Tramtrack-Bric a brac) or POZ (poxvirus and zinc finger) domain (Bardwell and Treisman, 1994; Chen et al., 1995; Collins et al., 2001; DiBello et al., 1991; Zollman et al., 1994). The BTB/POZ domain functions in inhibiting DNA binding and nuclear localization (Bardwell and Treisman, 1994; Collins et al., 2001). The BTB domain also has a role in substrate recognition for ubiquitin ligase (van den Heuvel, 2004; Xu et al., 2003). Additionally, the BTB/POZ domain allows for potential protein-protein interactions causing the formation of homodimers and heterodimers. In the multimeric form, BTB/POZ domains bind to different DNA sequences than the sequence recognized by a single BTB/POZ domain, which allows for diversity in regulation of transcription of target genes (van den Heuvel, 2004). In zinc finger transcription factors such as BR-C, the BTB/POZ domain helps regulates the zinc finger recognition of DNA binding sites especially in genes where there are multiple binding sites for the different BR-C isoforms.

The carboxy terminus of the six isoforms of the BR-C is composed of one of four pairs of C2H2 zinc fingers: BRC-Z1, BRC-Z2, BRC-Z3, and BRC-Z4. BRC-Z1 has three protein products from the BR-C that differ by the linker region between the core region and the zinc fingers. There is a high degree of sequence similarity in the four zinc finger pairs encoded by the BR-C. The variation in sequence between the BR-C isoforms occurs in the first zinc finger of each pair, which may be responsible for site-specific binding to cis-regulatory regions of DNA.

BR-C transcripts are found in all larval tissue, but the relative abundance of each transcript varies between tissue and developmental stages (Huet et al., 1993; von Kalm et al., 1994). In the salivary gland, BRC-Z1 expression fluctuates with time: increasing
during mid-third instar larval stage, peaking and decreasing at late third instar larval stage, and increasing during the prepupal stage (Huet et al., 1993; von Kalm et al., 1994). BRC-Z2 expression is barely detectable in the salivary glands until the prepupal stage where BRC-Z2 expression levels reach a maximum and then begin to decrease. BRC-Z3 expression in the salivary glands increases gradually over time with expression level beginning to increase in the mid-third instar larva stage. This variation of timing between expression of BR-C isoforms during metamorphosis is not just limited to the salivary glands but is also seen in other tissues. BRC-Z1 is expressed abundantly in the prepupal gut, salivary gland, and malpighian tubules with BRC-Z1 levels decreasing with time. There are low levels of BRC-Z1 in the fat body and wing discs. The wing discs have the highest abundance of BRC-Z2. BRC-Z3 is in malpighian tubules, gut, and fat body. The abundance of BR-C isoforms in tissues is dependent upon the ecdysone concentration, promoter used for transcription, and regulatory regions located in the introns (Bayer et al., 1996; Gonzy et al., 2002; Karim and Thummel, 1992).

The BR-C contains four promoters: P_{120}, P_{163}, P_{165}, and P_{167} (fig 1.2) (Bayer et al., 1996; DiBello et al., 1991). The naming of the promoters is based on of the location of the promoter on the X-chromosome with the P_{120} promoter being 120kb from the tip of the X-chromosome. Northern analyses on RNA derived from imaginal discs with a probe directed towards exons that map to the P_{120} and P_{165} region, suggest that the P_{120} and P_{165} are the only functional promoters for BR-C in imaginal discs (Bayer et al., 1996). The non-functional P_{167} promoter was uncovered using RNAse protection experiments. The RNAse protection assay does not distinguish between fully processed, partially processed, or unprocessed RNA. Transcripts theoretically from the P_{167} promoter may be
the result of RNAse protection of partially processed RNA or the P_{167} promoter may be rarely used or not used in regulation of BR-C transcript expression in larva (Bayer et al., 1996). The lack of transcripts from P_{163} promoter may due to the fact that the P_{163} promoter was found with the BRC-Z4 isoform in a cDNA library and is the result of an incomplete cDNA (Bayer et al., 1996). In Northern analyses, the fully processed transcripts of the BRC-Z2, BRC-Z3, and BRC-Z4 isoforms are undetectable. Probing the transcripts with probes directed towards introns show that many of the large transcripts seen are due to incomplete splicing events (Bayer et al., 1996). This suggests that the regulation of BR-C transcripts is not just transcriptional but also post-transcriptional. Post-transcriptional modifications of a transcript, such as splicing, are a common way to regulate expression of the gene product. Northern blot analyses have revealed BR-C molecules that are larger than mature RNAs for each BR-C isoform (Bayer et al., 1996). The processing of the RNA may be the rate-limiting step of BR-C protein expression.

There are other cis-regulatory regions for the BR-C besides the four promoters already mentioned. One cis-regulatory region lies before the P_{120} promoter and two others are found after the P_{165} promoter (Bayer et al., 1996; Gonzy et al., 2002). The br^{28} mutant is caused by a P-element insertion into the BRC-Z2 DNA binding domain. Precise excision of this P-element does not fully revert the animal to wild type br\textsuperscript{+} function, leading investigators to discover a small deletion before the P_{120} promoter which has mutant effects (Gonzy-Treboul et al., 1995; Hodgetts et al., 1995; O'Keefe et al., 1995; Sampedro et al., 1989). This suggests that a cis-regulatory element of BR-C lies upstream to the P_{120} promoter. This result does not show where the regulatory region is located: the spacing could be altered. Imprecise excision of a P-element that lies 6.5kb
downstream of the P165 promoter and 4.5kb upstream of the core exon, gave rise to two mutants that did not complement br or 2bc mutants (Gonzy et al., 2002). The exact sequence of the regulatory domains remains to be determined. These regulatory domains may either regulate transcription of the BR-C isoforms or help regulate the BR-C postranscriptionally by providing stability for the transcript.

There is a relationship between BR-C protein isoforms and genetic functional redundancy (Bayer et al., 1997). Rescue of the br5, rbp1, and 2bc1 lethal mutants (table 1.1) with heat inducible transgenes of each of the four BR-C zinc finger isoforms was done to determine the role of the BR-C zinc finger in BR-C function. Heatshock induction of BRC-Z2, but not the other BR-C zinc fingers, rescues the lethal phenotype of br5. This outcome is consistent with a model in which defects in BRC-Z2 is responsible for the br5 lethal phenotype. Rescue of the lethal phenotype of rbp1 was accomplished with the heatshock induction of BRC-Z1 or BRC-Z4 (Bayer et al., 1997). Induction of BRC-Z1 had the strongest rescues of rbp1 lethality rescuing 58% of rbp1 larvae to adulthood, whereas induction of BRC-Z4 only rescues 31% of rbp1 larvae to adulthood. A defect in BRC-Z1 is responsible for rbp1 lethality. The partial rescue of rbp1 lethality with overexpression of BRC-Z4 suggests that BRC-Z4 is a weak but functionally equal form of BRC-Z1. In addition, the lethality of 2bc1 is rescued by overexpression of BRC-Z3, which results in 71% of 2bc1 larvae surviving to adulthood. The 2bc1 lethality is also rescued to lesser extent with heatshock induction of BRC-Z2, which rescues 29% of 2bc1 larvae to adults. Loss of BRC-Z3 is primarily responsible for the 2bc1 phenotype, but BRC-Z2 has functional redundancy with BRC-Z3. From the evidence presented, the different mutant groups of BR-C do not each correspond to just one BR-C transcript.
<table>
<thead>
<tr>
<th>Mutant Group</th>
<th>Number of Alleles</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weak Mutant</td>
</tr>
<tr>
<td><em>broad (br)</em></td>
<td>29</td>
<td><em>br</em>&lt;sup&gt;l&lt;/sup&gt;- broadening of the wings, viable</td>
</tr>
<tr>
<td><em>reduced bristles on the palpus (rbp)</em></td>
<td>6</td>
<td><em>rbp</em>&lt;sup&gt;2&lt;/sup&gt;-reduced bristles, viable</td>
</tr>
<tr>
<td><em>2bc</em></td>
<td>5</td>
<td><em>2bc</em>&lt;sup&gt;l&lt;/sup&gt;-delayed puparium of 6 hours, pupal lethal</td>
</tr>
<tr>
<td><em>non-pupariating (npr)</em></td>
<td>8</td>
<td><em>npr</em>&lt;sup&gt;2&lt;/sup&gt;-pupal lethal</td>
</tr>
</tbody>
</table>

**Table 1.1 Alleles of the BR-C.** The table above describes the three complementing allele groups and the fourth group of non-complementing alleles. The number of alleles of each group is given along with the phenotypic description of a weak and strong allele of each group.
Although $br^+$ function is solely derived from BRC-Z2, disruption of $br^+$ expression does not just alter expression of BRC-Z2. The $br^{28}$ mutant demonstrates this. $br^{28}$ is a P-element insertion into the DNA binding domain of BRC-Z2 yet it alters both BRC-Z3 and BRC-Z1. BRC-Z3 transcript and protein levels are reduced in $br^{28}$ and the BRC-Z1 protein is truncated suggesting a more complex relationship between BR-C isoforms (Gonzy-Treboul et al., 1995).

1.2.1 The Role of BR-C in Metamorphosis

The BR-C plays a critical role in the hierarchy of the ecdysone signal. The transcription of late genes in morphogenesis requires the ecdysone-induced expression of the transcription factors BR-C, E74 and E75. The ecdysone signal is propagated first by the expression of BR-C. Induction of BR-C expression by ecdysone is then followed by the induction of E74 and E75. BR-C expression is necessary for the maximum induction of E74 and E75 suggesting that the BR-C is a key mediator of the ecdysone signal (Karim et al., 1993, Karim, 1992 #53). In particular, the 2bc+$^+$ transcripts (BRC-Z3) are required for maximal early gene induction.

In addition to mediating the ecdysone signal for induction of early genes, the BR-C plays an important part in regulating the transcription of late genes (Buszczak et al., 1999; Dubrovsky et al., 2001; Huang and Orr, 1992). The BR-C coordinates transcription of late genes in an ecdysone dependent manner at different times and in different tissues depending on the abundance of the BR-C zinc finger isoforms present. These late genes include the small heat shock genes clustered at 67B (such as shsp 23), and other late genes such as dopa decarboxylase (DdC), intermolt genes (Sgs-3, -4, -5), fat body protein 1, fat body protein 2, six genes in the L71E cluster, and let-7 (Dubrovsky et al., 1996,
Dubrovsky, 1994 #50, Mugat, 2000 #1, Sempere, 2002 #47, Crowley, 1984 #54; Dubrovsky et al., 2001; Hodgetts et al., 1995; Karim et al., 1993). All of these late genes do not express in a BR-C null mutant and each late gene requires the expression of certain BR-C zinc fingers for expression (Dubrovsky et al., 1996, Dubrovsky, 1994 #50, Mugat, 2000 #1, Sempere, 2002 #47, Crowley, 1984 #54; Dubrovsky et al., 2001; Hodgetts et al., 1995; Karim et al., 1993).

The BR-C is required for ecdysone dependent shsp23 expression during metamorphosis. Mutation in the BR-C reduces the level of shsp23 transcripts by 95 to 99% of wild type levels (Dubrovsky et al., 1994, Dubrovsky, 1996 #7). The different isoforms of BR-C regulate expression of shsp23 in a tissue specific manner (Dubrovsky et al., 1996). 2bc mutants (mutants of BRC-Z3) have low levels of shsp23 transcripts in the fat body and the malpighian tubules with no effect on expression of this gene in the salivary glands. br mutants (mutants of Z2) have no effect on shsp23 transcript expression in the malpighian tubules and the fat body, but proper br+ function is absolutely necessary for shsp23 expression in wing discs and midgut tissues (Dubrovsky et al., 1996). Mutants of rbp (Z1 mutants) have no effect on expression levels of shsp23.

BR-C regulates ecdysone dependent shsp23 expression by binding to 5 regulatory sites in the shsp23 locus (Dubrovsky et al., 1996, Dubrovsky, 2001 #6). These five sites consist of nine to twelve base pairs with a central core. The core is 3bp that determine which isoform binds the regulatory site (Dubrovsky et al., 1996; von Kalm et al., 1994). Thus, the core element provides isoform-binding specificity, yet this cannot be sufficient because of mutant data showing functional redundancy between the BR-C isoforms. Of the five regulatory sites, the fifth site is critical for ecdysone regulated shsp23 expression.
(Dubrovsky et al., 2001). Three of the *BR-C* isoforms, BRC-Z1, BRC-Z2, and BRC-Z3, bind to this fifth site with BRC-Z2 having the highest binding affinity for the site indicating that *br* function is important for ecdysone dependent *shsp23* gene expression (Dubrovsky et al., 1996; Dubrovsky, 2001 #6).

Like *shsp23*, *Dopa Decarboxylase (Ddc)* requires proper *BR-C* function for expression during metamorphosis (Hodgetts et al., 1995; O'Keefe et al., 1995). During metamorphosis, *Ddc* is expressed in both the neurons and the epidermis. The *Ddc* expressed in the epidermis is a splice variant of the *Ddc* expressed in the neurons. The *BR-C* binds to a cis-acting region that is required for tissue specific splicing of *Ddc* (Hodgetts et al., 1995). *br* has a five-fold decrease in total *Ddc* expression levels in larvae, while *Ddc* expression levels are unaltered in both the *rpb* and *2bc* mutants compared to wild type siblings (Hodgetts et al., 1995; O'Keefe et al., 1995). Heatshock induction of BRC-Z2 in a *br* background rescues *Ddc* expression to near wild type levels while heat shock induction of BRC-Z1, BRC-Z3, and BRC-Z4 does not (Bayer et al., 1997). This suggests that the BRC-Z2 isoform is the key *BR-C* isoform for proper expression of *Ddc*. The *Ddc* gene has six cis-acting regions, which are bound by at least one of the four zinc finger pairs of the *BR-C*. As seen with *shsp23*, the cis-acting region of *Ddc* that BRC-Z2 binds contains the central core sequence essential for BRC-Z2 binding (Dubrovsky et al., 1996; O'Keefe et al., 1995; von Kalm et al., 1994). Although BRC-Z2 is the primary isoform required for *Ddc* expression, the fact that BRC-Z1, BRC-Z3, and BRC-Z4 are capable of binding to the gene indicates that they may play a regulatory role in tissue specific splicing of *Ddc*. 
BRC Z1 and BRC-Z4 are necessary for the induction of the intermolt genes, salivary gland secretion proteins 1,2,3,4 (sgs-1, 2,3,4) (Bayer et al., 1997, Guay and Guild, 1991, Crowley et al., 1984). There are 6 cis-acting regulatory sites in the sgs gene cluster; the BR-C binds to four of these sites (von Kalm et al., 1994). In rbp mutants, the expression levels of sgs-3, -4, -5 are 3%, 10%, and 33% respectively of wild type levels (Guay and Guild, 1991). The reduced expression of the sgs genes is rescued fully by heatshock induction of BRC-Z1, and partially rescued by induction of BRC-Z4 (Bayer et al., 1997). This rescue of sgs gene expression is not seen with overexpression of BRC-Z2 or BRC-Z3.

Two cis-regulatory elements are required for BR-C induced fat body protein 1 (fbp1) expression, (Mugat et al., 2000). These elements are known as the Amplifying and Enhancing (AE) elements. A transgenic construct with LacZ under the control of the AE element (AE-LacZ) was used to determine which BR-C isoform is necessary for fbp1 expression. Heatshock-induced expression of BRC-Z1, BRC-Z3, and BRC-Z4 induce expression of the AE-LacZ transgene in a BR-C null mutant demonstrating a functional redundancy of BRC-Z1, BRC-Z3 and BRC-Z4. Heatshock induced expression of BRC-Z2 represses the expression of the AE lacZ construct. Mugat et al. found that the expression levels of the various BR-C isoforms vary in the third instar larva, with BRC-Z2 having the highest concentration at the beginning of the third instar larva and decreasing in mid-third instar larva (Mugat et al., 2000). Late in the third instar, BRC-Z2 becomes undetectable, while BRC-Z1, BRC-Z3, and BRC-Z4 are highly expressed. AE-LacZ expression fluctuates with the fluctuation in BR-C isoform concentrations. This
suggests that BR-C isoforms regulate fat body 1 protein expression by binding cis elements and by concentration of the isoform.

After the ecdysone pulse and the induction of BR-C, E74, and E75, a set of six late genes located at L71E are induced (Crossgrove et al., 1996, Guay and Guild, 1991). These genes are known as L71E-I, II, III, IV, V, and VI. The inductions of the L71E genes are dependent on rbp^+ function (BRC-Z1) of the BR-C. The level of L71E genes I, II, V and VI in rbp^I are reduced to 3% of wild type levels, while L71E gene III and IV in rbp^I are decreased to 10% of wild type levels (Guay and Guild, 1991). Other BR-C mutants except the null mutants have wild type levels of L71E genes. Heat shock induction of the BRC-Z1 isoform rescues L71E gene expression, while induction of BRC-Z2, BRC-Z3, and BRC-Z4 do not rescue L71E gene expression in BR-C null mutants (Crossgrove et al., 1996). It was also found that L71E is down regulated by induced expression of BRC-Z3 and BRC-Z4 in wild type flies (Crossgrove et al., 1996) suggesting a regulatory role for BRC-Z3 and BRC-Z4 in L71E gene expression. As described earlier, L71E also contains the conserved cis-acting element to which BRC-Z1 binds.

let-7 is also dependent upon proper BR-C function for expression (Sempere et al., 2002). let-7 is a small regulatory RNA, which represses translation of lin4 by binding to the 3’ UTR of lin4 RNA. let-7 first appears at the end of the third instar larval stage and reaches its highest level of expression during pupal development (Hutvagner et al., 2001; Pasquinelli et al., 2000). In BR-C null mutants and in BR-C RNAi, expression of let-7 is low (Sempere et al., 2002). The BR-C zinc fingers that regulate let7 remains to be determined. As one can surmise from the information provided above, there is a complex
relationship between the BR-C isoforms in regulating the expression of downstream targets. In addition to regulating ecdysone signals in morphogenesis, the BR-C plays a role in oogenesis.

1.2.2 The Role of BR-C in Oogenesis

In Drosophila, oogenesis can be divided into 14 stages of development. BR-C protein is detected in egg chamber development during mid oogenesis, at stage six through stage 10A, in the nuclei of follicle cells (Buszczak et al., 1999; Deng and Bownes, 1997; Huang and Orr, 1992; Tzolovsky et al., 1999). The presence of BR-C is not detectable in the germline (Buszczak et al., 1999; Deng and Bownes, 1997). Since BR-C is induced by ecdysone during metamorphosis, it is reasonable to think that ecdysone induces BR-C expression during oogenesis because BR-C expression during oogenesis is reduced in ecdysone mutants (Buszczak et al., 1999).

Misexpression of BR-C affects DNA replication and chorion development during oogenesis (Huang and Orr, 1992; Tzolovsky et al., 1999). Overexpression of BR-C isoforms increases DNA replication (Tzolovsky et al., 1999), while BR-C hypomorph mutants have a decrease in DNA replication (Huang and Orr, 1992). In addition, chorion development is altered when BR-C is misexpressed. This is best observed in the abnormal dorsal appendages of the egg when BR-C levels are either decreased or increased relative to wild type levels. Non-lethal rbp mutants have abnormal or missing dorsal appendages, filaments located at the anterior-dorsal region of the egg that mark both the dorsal-ventral and anterior-posterior polarity of the eggshell, suggesting a role for rbp in appendage formation (Deng and Bownes, 1997). Heatshock induction of BRC-Z1 causes an increase in the number of dorsal appendages of wild type flies. Of all of the
isoforms, BRC-Z1 is the most abundant in wild type ovaries suggesting that \( rbp^+ \) function is necessary for oogenesis (Deng and Bownes, 1997). Increased number of dorsal appendages are also seen when BRC-Z2, BRC-Z3, and BRC-Z4 are induced by heatshock, yet these isoforms are not abundantly expressed in oogenesis suggesting that the BR-C isoforms work in a complex manner (Deng and Bownes, 1997).

1.3 Hypothesis

The role and function of the \( BR-C \) has been thoroughly studied in development and metamorphosis. \( BR-C \) is known to have a role in oogenesis but the details of the \( Br-C \) actions in oogenesis remain unclear. \( BR-C \) is expressed during oogenesis and has been detected in the follicle cells of the ovaries, but not much more is known about the function of the \( BR-C \) in adults. As stated earlier, there is a complex functional interaction between the \( BR-C \) isoforms. In some mutant cases, one isoform is necessary for complete expression of a given gene but that gene expression can be partially rescued by another isoform: this is observed in \( BR-C \) induction of the intermolt genes. In other cases, one \( BR-C \) zinc finger induces expression of a target gene while another \( BR-C \) zinc finger represses expression of the same gene. An example of this is the expression of the L71E gene cluster. The complexity of the \( BR-C \) in gene regulation can be demonstrated in the multiple DNA binding sites of \( shsp23 \) where there are five cis-regulatory regions that the different \( BR-C \) isoforms can bind. The expression of \( BR-C \) genes is both transcriptionally regulated and postranscriptionally regulated. Understanding the known functions of \( BR-C \) may aid in deciphering the role of \( BR-C \) in mediating stress response.

We are interested in studying molecular pathways that alter lifespan and stress response in adult \textit{Drosophila melanogaster}. As mentioned in section 1.1, lifespan
appears to be under control of the endocrine system by two pathways: the insulin-signaling pathway and a steroid-signaling pathway. Key members of each pathway have been shown to modify lifespan in *D. melanogaster*, yet a full picture of how each pathway regulates aging remains to be determined. Our focus is studying the involvement of the ecdysone steroid pathway in aging and elucidating key members of the steroid aging pathway in *D. melanogaster*.

It has been shown that a decrease in ecdysone levels or a decrease in the ecdysone signal by mutations in the ecdysone receptor leads to an increase in stress resistance and extended lifespan in *D. melanogaster* (Simon et al., 2003). Lowered expression of ecdysone in *D. melanogaster* leads to lowered expression of the *BR-C* (Buszczak et al., 1999). I have discovered a long-lived and stress resistant fly line (c754) that has altered *BR-C* expression. I hypothesize that *BR-C* regulates stress resistance and lifespan. I tested this hypothesis three ways: further examination of the *BR-C* misexpressing mutant c754, heatshock overexpression of the *BR-C* isoforms, and underexpression of the *BR-C* isoforms by RNAi.
Chapter 2: Materials and Methods

2.1 Fly Cultures

For most experiments, flies were raised at room temperature on standard cornmeal agar media. For analysis of the effect of varying calories on lifespan experiment, flies were raised on standard sucrose yeast media at room temperature. Sucrose yeast media contains 10% sucrose, 10% yeast, 0.3% propionic acid, 3% nipagin, and 2% agar in water. Flies were shifted to 0.65X, standard (1X), and 1.5X sucrose yeast media (Table 2.1). For all experiments, flies were placed at a density of ten mating pairs per bottle. Parents were cleared after five days of mating.

2.2 Fly lines

2.2.1 c754

c754 is a Gal4 expression line that was obtained from the Drosophila stock center at Bloomington, IN and originally constructed by J. D. Armstrong in the lab K. Kaiser. The Gal4 expression pattern of c754 which sparked our interest was done in the lab of Norbert Perrimon (Harrison et al., 1995). Excision lines, (isogenic lines to c754) were obtained by mobilizing the P-element using Δ2-3Dr transposase (fig. 2.1). c754 females were crossed to males possessing the Δ2-3 transposase marked with the selectable marker drop. Male progeny were selected for their Drop eye phenotype and mated to an attached X female. Male progeny from this cross receive their X chromosome from their father and their Y chromosome from their mother. Male progeny from the attached X cross were selected for their eye color and shape. Red eye color means the P-element is still in
<table>
<thead>
<tr>
<th></th>
<th>0.65X</th>
<th>1X</th>
<th>1.5X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>15g</td>
<td>20g</td>
<td>20g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>65g</td>
<td>100g</td>
<td>150g</td>
</tr>
<tr>
<td>Yeast</td>
<td>65g</td>
<td>100g</td>
<td>150g</td>
</tr>
<tr>
<td>Nipagen</td>
<td>30ml</td>
<td>30ml</td>
<td>30ml</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>3ml</td>
<td>3ml</td>
<td>3ml</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1 liter</td>
<td>1 liter</td>
<td>1 liter</td>
</tr>
</tbody>
</table>

Table 2.1 Ingredients for caloric restriction media. To make food of altering caloric content measure the ingredients for the desired food. Add all ingredients, except the nipagen and propionic acid, to a final volume of one liter. Bring the mix slowly to a boil, stirring constantly. Once food has reached a boil, stir until it thickens. Remove from heat add nipagen and propionic acid, stir.
Figure 2.1 c754 excision cross. 1) c754 virgin females are crossed to males possessing the Δ2-3 transposase marked with drop. Cross 1) is a mass mating while all other crosses described are single matings. Male progeny from cross 1) with Drop eyes were selected for cross 2). Cross 2) males have a mobilized P-element (c754) and are crossed to attached X females. Attached X females have a Y chromosome, which is donated to male progeny. Male progeny from cross 2) that retained red-eyes and the Drop phenotype were used in cross 3) and mated to attached X females. Cross 3) retained the transposase to use the selectable third chromosome marker Drop for elimination of any of the original c754 third chromosome. 4) Non-Drop Red-eye and white-eye male progeny from cross 3) were mated to attached X females for bulking up the males for sequence analysis of the transposition event. The red-eye progeny are putative c754 males and the white-eye progeny are putative excisions of c754. The excision can be imprecise or precise. To determine the type of excision white-eye male genomic DNA was sequenced. To determine retention of the P-element in the original c754 location in red-eye males, genomic DNA was sequenced.
the genome and white-eye color means that either an excision of the P-element occurred or the mini-\textit{w} that is used to select for the P-element was silenced. Males with normal shaped eyes indicate that the transposase is not in the genome. Single males with normal eyes that were either red or white were selected and crossed to attached X females for maintenance of the line. From these lines, males were selected for determining the precision of the excision on white eyed flies and location of the P-element in red eyed flies. P-element excision sometimes is imprecise either deleting some of the chromosomal DNA, or leaving part of the P-element DNA behind. To determine the precision of the excision, a PCR based analyses was done on genomic DNA from the white-eyed males. For the white eyed excised lines, PCR primers, c754-L and c754-R, were designed that would give a 1 kb product for a precise excision (Table 2.2). It is possible that the P-element was removed in red eye flies and reinserted into the genome. To determine the location of the P-element in red eye flies, new primers, P.R. attached and c754R were used (Table 2.2). Once excision or placement of the P-element was determined, males were crossed to an FM7 female to create a stock line with both males and females having the same X-chromosome.

### 2.2.2 HS \textit{BR-C} isoforms

Cynthia Bayer (Bayer et al., 1997) generated the HS-BR-C isoforms lines. Each fly line is homozygous for one of the \textit{BR-C} isoforms under control of the heatshock protein 70 promoter. Fly lines were verified for their insert by PCR. The forward primer used for testing all heatshock lines was RT-core (Table 2.2). The RT-BR-Z1, RT-BR-Z2, RT-Br-Z3, RT-BR-Z4 primers were used as the reverse primer to determine insertion of
<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>c754 L</td>
<td>TTC GTA CGA CTT TGC AAT TTG CAG TTC</td>
</tr>
<tr>
<td>c754 R</td>
<td>ATA TAT GCG GAC TTA CAG TTT CGG TCG</td>
</tr>
<tr>
<td>P.R. ligated</td>
<td>AAG AGC GCC CAA TAC GCA</td>
</tr>
<tr>
<td>P.R. attached</td>
<td>ACA AGC AAA CGT GCA CTG</td>
</tr>
<tr>
<td>RT-Br-Core</td>
<td>ACA AGA TGT TCC ATG CAG CC</td>
</tr>
<tr>
<td>RT-Br-Z1</td>
<td>TGC TGG TGC TGC TGG TGA TA</td>
</tr>
<tr>
<td>RT-Br-Z2</td>
<td>TCA TCT CCA TTT CGC CGG GA</td>
</tr>
<tr>
<td>RT-Br-Z3</td>
<td>GAT GGC GGT CTT AAG CA</td>
</tr>
<tr>
<td>RT-Br-Z4</td>
<td>GTG GTT GTT CAG CGA GTT CA</td>
</tr>
<tr>
<td>RT-RpL27-L</td>
<td>GAA TAT CAA GCG GAA GAA GAC CAG GAA G</td>
</tr>
<tr>
<td>RT-RpL27-R</td>
<td>AAT TTA ACC AAG TCG ATG ACG GGA GC</td>
</tr>
<tr>
<td>Acc65 Z1</td>
<td>ACA GGT ACC CCC CCC CCC CGA TTT TGT TTT</td>
</tr>
<tr>
<td>Nhe1 Z1</td>
<td>ACA GCT AGC CAC TAC TGT TAG CTC CTT ATT AAG ACG AC</td>
</tr>
<tr>
<td>Br-core-RNAi L</td>
<td>CAG AAT TCA ATC CGG CAA GCG GTC TAG TAA TCA</td>
</tr>
<tr>
<td>Br-core-RNAi R</td>
<td>CAG AAT TCA CGA CGG GGC GTT TGA TG</td>
</tr>
<tr>
<td>Br-Z1-RNAi L</td>
<td>CAG AAT TCA GCA AAT GTA CGC GGC GTT GAT G</td>
</tr>
<tr>
<td>Br-Z1-RNAi R</td>
<td>CAG AAT TCT TGC GTT AGA TGC TCT GTG GTG T</td>
</tr>
<tr>
<td>Br-Z2-RNAi L</td>
<td>CAG AAT TCA AGA AAC TCT TCT CAT GCC AGC TCT GC</td>
</tr>
<tr>
<td>Br-Z2-RNAi R</td>
<td>CAG AAT TCG GGA CGG GAC TTG TGG TAC GTG TA</td>
</tr>
<tr>
<td>Br-Z3-RNAi L</td>
<td>CAG AAT TCC ACC AGC TGC TGC ATA AAC GAG CC</td>
</tr>
<tr>
<td>Br-Z3-RNAi R</td>
<td>CAG AAT TCT TCA GCA TGC CGC TAG AGC CG</td>
</tr>
<tr>
<td>Br-Z4-RNAi L</td>
<td>CAG AAT TCT GCC CAG GCC AAT AGA CTC CTC CT</td>
</tr>
<tr>
<td>Br-Z4-RNAi R</td>
<td>CAG AAT TCT TCT GCC GGC GAT GAG TGA TGC TCT T</td>
</tr>
<tr>
<td>SacII Gal4F</td>
<td>CAC CGG CGG ATG AAG CTA CTG TCT TCT ATC GAA CAA GC</td>
</tr>
<tr>
<td>SacII Gal4R</td>
<td>CAC GAG CTC GTT ACA TAA AAG AAG GCA AAA CGA TG</td>
</tr>
<tr>
<td>F-RpL27 REAL</td>
<td>GGT CGG AGC CGA GAA GTT</td>
</tr>
<tr>
<td>RpL27-R2</td>
<td>GGC CCA GCA GCT TGT AGT AG</td>
</tr>
<tr>
<td>BR-Z1-L2</td>
<td>AGG AGC CCG TAA GCT CCT CT</td>
</tr>
<tr>
<td>BR-Z1-R2</td>
<td>TTG AGG TTG CGG TGG TAG AT</td>
</tr>
<tr>
<td>BR-Z2-R1</td>
<td>GCT CTG CGG TAA GCT CCT CT</td>
</tr>
<tr>
<td>BR-Z2-R1</td>
<td>GAG TGG CGG GAG CAG TAG AC</td>
</tr>
<tr>
<td>CORE REAL TIME</td>
<td>CCT CAG CGT TGC TAG GTC</td>
</tr>
<tr>
<td>REAL TIME BR-Z3</td>
<td>TGG CGA TGA GGT AGA ACT GGT</td>
</tr>
<tr>
<td>Z4 REAL TIME</td>
<td>ACG CCA CTG AGT AGG CTG TC</td>
</tr>
</tbody>
</table>

Table 2.2 Primers used. Primers are listed 5' to 3'.
BRC-Z1, BRC-Z2, BRC-Z3, and BRC-Z4 respectively (Table 2.2). To create a proper control for the HS-BRC-transgenic lines, homozygous transgenic flies were crossed to w<sup>1118</sup>, the fly line used for creation of these transgenic flies. Progeny from this cross would be heterozygous for the transgene. The heterozygous progeny were then allowed to mate, which would give rise to three genotypes: homozygous BR-C transgenic flies, heterozygous BR-C transgenic flies, and non-transgenic flies. This procedure generates flies with the same randomized genetic background with the only difference being the amount of transgene in the fly.

### 2.2.3 Construction of UAS-BR-C lines

Plasmids containing the cDNA for BRC-Z1, BRC-Z2, BRC-Z3 and BRC-Z4 were obtained from Cynthia Bayer. UAS constructs were made by cloning the cDNA of interest into the pUAST vector. The pUAST vector is described in Brand et al. (Brand and Perrimon, 1993).

The BRC-Z1 cDNA was inserted into the EcoR1 site of pBluescribe M13+ plasmid. Primers were generated that would amplify the cDNA and engineer an Acc65I site at the 5' end and an NheI site at the 3' end onto the BRC-Z1 cDNA. The primers are Acc65I-Z1 and NheI-Z1 (Table 2.2). The PCR product was purified with the Mo Bio PCR clean up kit and cut with Acc65I and NheI. The cut BRC-Z1 PCR fragment was ligated into the pUAST vector that was cut with Acc65I and XbaI with T4 DNA ligase. NheI and XbaI create compatible cohesive ends. This is a directed ligation so BRC-Z1 inserts into the pUAST vector in the proper orientation. However, despite repeated
attempts, I was never successful at generating transgenic UAS-BRC-Z1 lines; instead, Dr. Riddiford provided a transgenic line with the pUAS-Z1 insert.

Cloning of BRC-Z2 and BRC-Z3 cDNA followed a similar scheme and will be discussed together. The BRC-Z2 and BRC-Z3 cDNAs were in the pBluescript plasmid. Flanking EcoRI sites were used to extract the BRC-Z2 and BRC-Z3 cDNAs from their initial vectors. Each cDNA was inserted separately into the EcoRI site of the pUAST vector. A BamHI digestion of the pUAS-BRC-Z2 and Z3 will determine the direction of the BRC-Z2 and BRC-Z3 cDNA insertion into the pUAST vector. Correct insertion of BRC-Z2 into pUAST would give three fragments of 671bp, 1.77 kb, and 2.078 kb when digested with BamHI. Correct insertion of BRC-Z3 into pUAST would give two fragments of 2.341kb and 2.258kb when digested with BamHI. After plasmids containing BRC-Z2 and BRC-Z3 correctly oriented in pUAST were identified, the plasmids were injected in w^{1118} embryos along with a helper plasmid that contains a hyperactive transposase (Beall et al., 2002). Injection of the plasmid was determined to be successful if some of the progeny from the injected line had eye colors that were some shade of red varying from light yellow to dark red.

To determine the chromosome in which the plasmid had inserted, flies with an eye color were mated to a balancer line that had white eyes and the markers CyO and Sco: CyO is a second chromosome balancer and Sco is located on the sister second chromosome. Balancer chromosomes do not recombine.

If the transgenic parent was male and all of the red-eyed progeny from the cross to w; CyO/Sco lines were female, then I concluded from this sex linkage that the transgene inserted into the X-chromosome (fig 2.2). If the transgene was not on the
Red-eyed transgenic male \[ X \ w^c; \text{CyO/Sco Bl}^+ \]

Only female progeny have red-eyes

\[ \text{Yes, then transgene inserted into X-chromosome} \]

\[ \text{No, cross red-eyed, curly wing males to w$^c$; CyO/Sco Bl}$^+ \text{ females} \]

Some red-eye progeny have both CyO and Sco Bl markers

\[ \text{Yes, transgene inserted on third chromosome} \]

\[ \text{No, transgene inserted on second chromosome} \]

**Figure 2.2 Determining chromosomal location of transgene insertion in transgenic males.** The transgene has the $w^+$ selectable marker and is inserted into a $w^c$ background. One can confirm insertion into the genome by eye color; some shade of red indicates transgene insertion. One can determine chromosomal location of insertion by tracking eye color through crosses. If the transgene is on the X-chromosome, then only female progeny from the transgenic male cross to $w^c$; CyO/Sco Bl$^+$ females will have red eyes. If both male and female progeny have red eyes, then the transgene inserted into either the second or third chromosome. After eliminating the possibility of insertion into the X-chromosome, red-eye curly wing males are mated to $w^c$; CyO/Sco Bl$^+$ females. If some of the red-eye progeny have both the CyO and Sco Bl markers, then the transgene inserted into the third chromosome. If red-eye progeny have either the CyO or Sco Bl marker then the transgene inserted into the second chromosome. CyO and Sco Bl are both second chromosome markers; red-eye progeny can only get both markers in if transgene is not on second chromosome.
X-chromosome, then male progeny were crossed to w'; CyO/Sco females to determine onto which autosome the transgene inserted. Red eye progeny that have both CyO and Sco markers indicated that the transgene inserted on the third chromosome, if either CyO or Sco markers but not both were in the red eye progeny then the transgene inserted on the second chromosome.

If the initial red eye transgenic parent were female, male progeny with CyO and red eyes from the w'; CyO/Sco cross would be selected and mated to w'; CyO/Sco females (fig. 2.3). If only female progeny had red eyes, then I concluded that the transgene inserted into the X-chromosome. If some red eye progeny had both CyO and Sco BI markers it was determined that the transgene inserted on the third chromosome. If red eye progeny had either CyO or Sco BI markers, then it was determined that the transgene inserted on the second chromosome. Homozygous UAS constructs for both BRC-Z2 and BRC-Z3 on all chromosomes were created.

Insertion of the BRC-Z4 cDNA into pUAST was accomplished by first excising BRC-Z4 cDNA from pBluescript using a scheme involving sequential and partial digestion. NotI and Acc65I sites flank the BRC-Z4 cDNA; however, BRC-Z4 has an internal NotI site. The pBS-BRC-Z4 was digested with NotI for 1 min and the enzyme was heat killed at 70°C for fifteen minutes. Acc65I was then added for a full digestion of the pBS-BRC-Z4 vector. This digestion gave a 3.8 kb, 3 kb and 800bp DNA fragment. The 3.8KB fragment is the desired BRC-Z4 fragment. The 3 kb band contains part of the BRC-Z4 cDNA that was cut at both the internal and flanking BRC-Z4 NotI site. The 800bp fragment is the remaining BRC-Z4 from the fully digested 3.8 kb BRC-Z4 fragment. The 3.8Kb fragment was inserted into the NotI and Acc65I sites of pUAST.
Red-eye transgenic female  \( X \)  \( w^+; \text{CyO/Sco Bl;} + \)

Red-eye, curly wing male progeny  \( X \)  \( w^+; \text{CyO/Sco Bl;} + \)

\[ \text{Only female progeny have red-eyes} \]

- Yes, then transgene inserted into X-chromosome
- NO, transgene inserted into second or third chromosome, do some red eye progeny from second cross have both CyO and Sco Bl markers

\[ \text{Yes, transgene inserted into third chromosome} \]
\[ \text{No, transgene inserted into second chromosome} \]

**Figure 2.3 Determining chromosomal location of transgene insertion in transgenic females.** The transgene has the \( w^+ \) selectable marker and is inserted into a \( w^+ \) background. One can confirm insertion into the genome by eye color; some shade of red indicates transgene insertion. One can determine chromosomal location of insertion by tracking eye color through crosses. Transgenic females are crossed to \( w^-; \text{CyO/Sco Bl;} + \) males. Red-eye curly wing male progeny are crossed to \( w^-; \text{CyO/Sco Bl;} + \) females. Eye color and phenotype of the progeny from this cross are used to determine chromosomal insertion of the transgene. If only female progeny have red-eyes, then the transgene inserted into the X-chromosome. If both male and female progeny have red eyes, then the transgene inserted into the second or third chromosome. If some red-eye progeny have both the CyO and Sco Bl marker then the transgene inserted on the third chromosome. CyO and Sco Bl both mark the second chromosome; red-eye progeny with both markers can only happen if the transgene is not on the second chromosome. If either the CyO or Sco Bl marker is present in red-eye progeny then the transgene inserted into the second chromosome. All crosses are single male to single female crosses.
pUAST-BRC-Z4 was injected into w\textsuperscript{1118} as described above. UAS-BRC-Z4 was found in multiple fly lines with inserts on all chromosomes.

2.2.4 Construction of BR-C RNAi Lines

To construct BRC RNAi plasmids, fragments from the BR-C cDNA plasmids were amplified and inserted into the pSymp-UAST vector using PCR. pSymp-UAST is described in Giordano et al (Giordano et al., 2002). The primers used were designed to amplify a specific region of each isoform and the core domain without cross-reaction to the other isoforms (Bayer et al., 1996). Each primer was engineered to have an EcoR1 site on the 5' end for easy insertion into the pSymp-UAST vector. The name of the primers used were Br-core-RNAi L and Br-core-RNAi R for the core fragment, Br-Z1-RNAi-L and Br-Z1-RNAi-R for the Z1 fragment, Br-Z2-RNAi L and Br-Z2-RNAi R for the Z2 fragment, Br-Z3 RNAi L and Br-Z3 RNAi R for the Z3 fragment, and Br-Z4-RNAi L and BR-Z4-RNAi R for the Z4 fragment (Table 2.2). The PCR products produced using these primer sets are 501 bp for the core, 320 bp for the BRC-Z1 isoform, 193 bp for the BRC-Z2 isoform, 220 bp for the BRC-Z3 isoform, and 229 bp for the BRC-Z4 isoforms. The PCR products were purified with the MoBio PCR Clean up kit and cut with EcoR1 restriction enzyme. The EcoR1 cut PCR product was ligated into EcoR1 site of the pSymp-UAST vector. The ligated reaction is then transformed into DH5α cells. The orientation of the insert into the plasmid is irrelevant because the plasmid is designed to read the insert from both sides of the insert. The pSymp-\textit{BR-C} isoform constructs were then injected into w\textsuperscript{1118} embryos as described above. Several single transgenic lines were isolated for each isoform and the core domain.
2.2.5 Construction of the Tet-O Gal4 Driver Line

Gal4 was cloned out of the pGawB plasmid using primers specific to the Gal4 gene. The primers used were SacIIGal4F and Sac1Gal4R (table 2.2). The forward Gal4 primer had a SacII site and the reverse primer had a SacI site. The SacI site is recognized by the restriction enzyme Ecl136I that gives a blunt end. The Gal4 PCR product was digested with SacII and Ecl136I and the Tet-O plasmid was digested with SacII and Hpal. Hpal leaves a blunt end. The digested Gal4 PCR product was ligated into the digested Tet-O plasmid. The ligated plasmid was transformed into DH5α cells. The Tet-O Gal4 construct was injected into w^{1118} embryos. Several Tet-O Gal4 lines were generated with Tet-O Gal4 on multiple chromosomes. No experiments were done with these lines, but they were generated in case an inducible Gal4 driver was needed for RNAi expression.

2.2.6 Expression of the RNAi Constructs with Either Act-5 Gal4 or Hs-Gal4

To express RNAi constructs from the pSymp-UAST-BRC transgenes, I crossed BRC RNAi transgenic flies to Act5-Gal4. The Act5-Gal4 driver is on the second chromosome balanced by CyO. The RNAi transgenic lines used in the cross were on the second chromosome. The RNAi lines for BRC-Z1, BRC-Z2, and BRC-Z4 were homozygous for the transgene on the second chromosome while the BRC-Z3 and core RNAi transgenic lines were heterozygous on the second chromosome balanced by CyO. RNAi virgin females were crossed to Act5-Gal4 males. Genotype of the progeny was determined by a wing morphology phenotype (curly or straight) and by eye color. Curly wings show the absence of Act-5 Gal4 or, in the case of heterozygote BRC-Z3 and core
RNAi lines, that either the Act-5 Gal4 was not present or the RNAi transgene was not present. Eye color was employed in examining progeny of the crosses of BRC-Z3 and core RNAi lines to Act-5 Gal4 to identify those flies with a darker shade of red, showing that both the RNAi construct and the Act-5 Gal4 driver. The pupal arrest observed in progeny from Act-5 Gal4 crossed to any of the BRC RNAi lines was not prevented when flies were grown at lower temperatures, such as 18° C.

The heatshock-inducible Gal4 driver (HS-Gal4) was also tested as a means to direct expression of BRC RNAi in adults. The HS-Gal4 line is homozygous for the HS-Gal4 transgene on the third chromosome. The same RNAi flies were used in this cross as in the previous Act-5 Gal4 cross. The progeny from the cross of HS-Gal4 males to RNAi BRC-Z1, Z2, and Z4 females were all heterozygous for both the HS-Gal4 driver and the RNAi transgene. The progeny from the cross of HS-Gal4 males and RNAi BRC-Core females were heterozygous for the HS-Gal4 driver and either the RNAi BRC-core transgene or CyO. RNAi BRC-Z3 was not crossed to HS-Gal4 because of difficulties in obtaining progeny from the available RNAi BRC-Z3 lines.

2.3 Phenotype Assays

2.3.1 Starvation Assays

For the non-heatshock starvation assays, cultures of c754 and its control were started at a density of ten mating pairs per bottle. After five days of mating, parental flies were discarded from the bottles. When progeny began to eclose, the bottles were cleared and progeny were allowed to eclose for two days. After two days, flies were anesthetized by CO₂, separated by sex, and placed 20 flies per vial, five vials per sex. The flies were
allowed to recover over normal cornmeal agar food for a minimum of one hour. After the one-hour recovery point, flies were put in vials that contained 3mls of 1% agar and placed at 18°C. The flies were checked every eight hours for death and the number that died were recorded. Death was measured as the absolute loss of movement.

For starvation assays with added heatshock, HS-\textit{BR-C} transgenes and the BRC-RNAi crossed to HS-Gal4 were used. Collection of flies was the same as described above. For the HS-\textit{BR-C}, flies were sorted by genotype based on eye color and then the genotyped flies were separated by sex. The homozygous flies have a deeper shade of red eyes than the heterozygous flies, and the no transgene flies have white eyes. For the HS-Gal4 RNAi crosses, RNAi females were crossed to both HS-Gal4 males and $w^{118}$ males. The cross to $w^{118}$ was done as a control line. In the RNAi crosses, the flies were separated by sex. Once separated by sex, all flies were placed in vials at a density of 20 flies per vial, five vials per sex. Flies were allowed to recover for a minimum of one hour. After recovery, flies were heatshocked in a 37°C incubator for one hour. After heatshock, flies were allowed to recover for eight hours at room temperature and then placed in vials that had 1% agar. Flies were kept at 18°C and checked for death every eight hours. Every 24 hours at the same time as their initial heatshock, flies were placed at 37°C for one hour. All starvation experiments went until complete death of flies was achieved.

2.3.5 Lifespan Assay in Demography Cages

For the cornmeal lifespan assay, flies were cultured on cornmeal agar food at a density of ten mating pairs in half-pint milk bottles. Parents were removed from the
culture after five days of mating and laying eggs. Progeny were allowed to emerge for two days and then collected and separated by sex. 300 flies per sex were placed in a demography cage. A demography cage is a hexagonal plastic jar with a mesh lid to allow air exchange and rubber gasket ports that hold a food vial and an aspiration tube for removing dead flies. There were three demography cages made per sex and genotype. Food was made and poured into wide-mouthed vials and allowed to solidify on a slant of 45° to create more surface area, and therefore more accessibility to the food for the flies. Flies were kept at 25°C with a twelve hour day night cycle. Food was replaced every two days. Dead flies were removed every other day and counted.

For the caloric restriction lifespan assay, flies were cultured on standard yeast media at a density of ten mating pairs per bottle. Parents were removed after five days. Progeny were allowed to eclose for one day and then placed in a new bottle containing standard sucrose yeast media for an additional two days. Flies were then collected and separated by sex. 300 flies per sex and genotype were placed in a demography cage. There were at least three demography cages per sex, genotype and food type. The flies were fed one of three types of food: 0.65X sucrose yeast media, standard sucrose yeast media, and 1.5X sucrose yeast media. All food was in wide vials and solidified on a slant. Food was changed every other day, when the dead flies were removed and counted. The cages were kept at 25°C with a twelve hour day night cycle.
2.4 Molecular Assay

2.4.1 Plasmid Rescue of c754

Genomic DNA was isolated from the c754 mutant and digested with either Acc65I or PstI. Both of these enzymes cut the transgene only once, and the enzymes do not cut in the ampicillin (Amp) resistant gene in the transgene. The digested DNA was allowed to circularize and ligate using T4 DNA ligase. The ligated DNA was transformed into DH5α cells and plated onto LB-Amp plates. Plasmids that had the transgene were amp resistant. Plasmids from bacterial colonies that were amp resistant were isolated and sent for sequencing. The sequencing primers are P.R attached and P.R. Ligated (Table 2.2). The sequence was compared to the *Drosophila* genome using a blast tool (Altschul et al., 1990).

2.4.2 Reverse Transcription PCR (RT-PCR)

RNA was isolated using a standard TriZol method, protocol provided by Invitrogen. The RNA was obtained from either whole flies or ovaries and carcasses. DNase treatment was not necessary because all primers used span an intron therefore; one can distinguish between RT amplification and genomic DNA amplification. The Superscript III first strand cDNA synthesis kit from Invitrogen was used to reverse transcribe 1μg of total RNA with the oligo dT primer provided with the kit. The Superscript III protocol provided was used for the reverse transcription reaction. The reverse transcription reaction was done in a PCR machine. 2μl of reverse transcript reaction were used for the PCR reaction. The forward primer for amplifying all the *BR-C* isoforms was RT-Br-core. The primers used for amplifying BRC-Z1, Z2, Z3, and Z4
were RT-Br-Z1, RT-Br-Z2, RT-Br-Z3, and RT-Br-Z4 respectively (Table 2.2) (Tzolovsky et al., 1999). Primers for detecting the housekeeping gene RpL27 were RT-RpL27-L and RT-RpL27 R (Table 2.2). The PCR was done using the Qiagen PCR kit with Q-solution. BRC-Z1 primers give three products: 974bp, 780bp, and 728bp. RT-PCR of BRC-Z2, BRC-Z3, and BRC-Z4 gives a 320bp fragment, 784bp fragment, and a 108bp fragment respectively. The RpL27 primers give a 315bp fragment.

2.4.3 Quantitative Real Time RT-PCR (Q-PCR)

RNA was isolated from whole females of approximately the same age using standard TriZol method. The RNA was then DNase treated using the DNA Free kit from Ambion following the company’s protocol. DNase treatment of the RNA was used to eliminate template contribution from genomic DNA. The reverse transcription reaction was carried out using the Superscript III first strand cDNA synthesis kit by Invitrogen: other RT reactions were tried but the Superscript III kit gave the best results. The company protocol was followed for the reverse transcription reaction. 5μg of total RNA was used for the reverse transcription reaction. 100ng of the newly transcribed cDNA was used for the Q-PCR reaction. The Eurogentics Q-PCR with syber green kit distributed by VWR was used for the detection of the amplified PCR product. The eurogentics kit is a master mix kit that contains everything necessary for Q-PCR except the cDNA and the primer. The total Q-PCR reaction was a total of 25μl with 12.5μl from the master mix, and the rest consisting of template, primer and water. Primers were used at 1μl each at concentration of 1.25 μM. The primers used for Q-PCR were: Br-Z1-L2 and BR-Z1-R2 for BRC-Z1, Br-Z2-L1 and BR-Z2-R2 for BRC-Z2, Core Real time and
real time BR-Z3 for BRC-Z3, Core real time and Z4 real time for BRC-Z4 and F-RpL27 real and RpL27-R2 for RpL27 (Table 2.2). The Applied Biosystem 7100 machine was used as the detection device for the Q-PCR reaction.
Chapter 3: The mutant c754

3.1 Background on c754 mutant

The identification of the c754 mutation was the result of follow up experiments on the *inebriated (ine)* mutant (Soehnge et al., 1996). *ine* is believed to have two functions: neurotransmitter transporter and osmolyte transporter. Mutation in the *ine* gene results in a hyperexcitable phenotype and an osmosensitive phenotype (Huang et al., 2002; Soehnge et al., 1996). Osmosensing in *Saccharomyces cerevisiae* is mediated by the Hog1/p38 pathway (Alexander et al., 2001; Brewster et al., 1993), which seems to be conserved from yeast to mammals as p38 is used in osmoregulation in mammalian tissue culture (Sheikh-Hamad et al., 1998). Mutations in the Hog1/p38 pathway often result in osmosensitivity, leading to the hypothesis that the osmosensitive phenotype of *ine* mutant flies is linked to the Hog1/p38 pathway. To test this hypothesis, vectors were created to express dominant negative p38 or constitutively active p38 mutants in the *ine* background. Expression for these vectors requires production of the yeast transcription factor Gal4. In testing Gal4 drivers for their background level of salt sensitivity, the novel c754 mutant was discovered.

The Gal4 driver line, c754, has increased resistance to salt, withstanding NaCl concentrations up to 700mM (fig 3.1). This is a dramatic increase over the control line S880, which can tolerate NaCl levels up to 500mM. The c754 mutation is caused by P-element insertion into the X-chromosome. This increased NaCl tolerance of the c754 mutant line intrigued me, leading to further characterization of the c754 mutant. First, the gene disrupted by the c754 insertion is the
Figure 3.1 *c754* is more resistant to osmotic stress. Mixtures of male and female flies from the *c754* or wild type, *s880* (wt), lines were tested for survival on fly food with NaCl. 80 flies (4 vials of 20 flies/vial) tested per condition. Experiment done by Claire Bocchini.
3.2 Determining the Gene Disrupted by the c754 P-element Insertion

Plasmid rescue was done to determine the location of the c754 insertion. Sequencing of the plasmids, determined that c754 is inserted into an introns of the Broad-Complex (BR-C) just after the P_{167} promoter and before the core exon, approximately 5Kb from the ATG start site (fig 3.2). As discussed earlier, there was at that time no known function of the BR-C in stress response. Because BR-C is induced by ecdysone signaling, our preliminary data suggested that the BR-C mediates ecdysone regulated stress response.

3.3 Stress and Lifespan phenotype in c754

In initial studies, stress resistance and longevity of the c754 mutant was compared to w^{118}, the line used to generate c754. However, comparison of c754 stress survival and lifespan to w^{118} stress survival and lifespan is not ideal because w^{118} is not in the same genetic background as c754. In order to characterize the stress tolerance and lifespan of c754, a proper control line was generated.

c754 is a P-element insertion line that has gone through many generations under unknown and varying selective pressures, which can induce and propagate mutations, thus changing the genetic background of the fly from w^{118}, the line used to construct c754. To construct a proper control for c754, the P-element inserted in c754 was precisely excised and verified by sequence analyses. Excision of the P-element allows the control line to have an X-chromosome that is isogenic to c754 only differing by excision of the P-element. Flies with a precise c754 excision and flies with c754 P-element still intact underwent three crosses in parallel to maintain the same genetic background (see fig 2.1). The crosses in parallel allow for comparison of both the
Figure 3.2 Schematic drawing of c754 insertion in the BR-C. c754 is located approximately 5 kb from the last promoter (green boxes) of BR-C and 5 kb from the transcription start site. White boxes are introns, orange boxes are UTRs.
excised c754 and the non-excised c754, in similar genetic backgrounds. In the experiments described below, c754-m is the non-excised fly line and Δc754-30 is the precise excision line. To determine whether c754-m retained the ability to survive longer than control during stressful conditions, lifespan between c754-m and Δc754-30 (control) during stressful conditions was measured.

C754-m flies lived longer under starvation conditions than Δc754-30 (control) flies. c754-m and Δc754-30 flies were collected and separated by sex and placed in vials containing 1% agar as a source for water. These data show that c754-m flies live longer during starvation conditions than the Δc754-30 line. Under starvation, c754-m females have a mean lifespan of 118 +/- 1 hour compared to 97 +/- 4 hours for Δc754-30 females (fig. 3.3). This is a 22% increase in mean lifespan in females (significant with a p-value < 0.05). c754-m males have a similar increase in survival under starvation conditions. The mean lifespan under starvation conditions for c754-m males and Δc754-30 males is 93 +/- 6 hours and 81 +/- 5 hours, respectively (fig. 3.4). c754-m males have a 16% increase in mean lifespan compared to the Δc754-30 under starvation conditions (significant with a p-value < 0.05). Testing c754-m for other stress tolerant phenotypes was not reproducible.

Because c754-m has an increased tolerance to starvation, we reasoned that the c754-m line might have an increase in lifespan. Lifespan studies took place in demography cages, which held 300 flies per cage. There were 3 to 4 cages per food, line and sex tested. Food was changed and death was measured every two days.
Figure 3.3 c754 females have an increase in starvation survival compared to the isogenic control Δc754-30. c754 (red) or control (blue) females were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The red and blue arrows are the mean starvation resistance of c754 and control respectively. \( t_2 = 5.02; \ p=0.04 \).
Figure 3.4 c754 males have increased starvation survival compared to the isogenic control, Δc754-30, males. c754 (red) or the control (blue) line were placed in vials containing 1% agar and the number of dead flies counted every 8 hr. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The red and blue arrows are the mean starvation resistance of c754 and control respectively. t2 = 5.80; p=0.03.
The c754-m mutation had different effects on lifespan of males and females raised on standard cornmeal agar food. c754-m females have a significant increase mean lifespan (48.0 +/- 2.0 days) compared to the Δc754-30 females (41.8 +/- 0.36 days) (fig. 3.5). In contrast, c754-m males (52.3 +/- 0.3 days) did not have a significant increase in mean lifespan compared to Δc754-30 males (48.2 +/- 3.0 days) (fig. 3.6). These results suggest that altering BR-C expression has an effect on female lifespan but not male. An increase in lifespan is observed in females but not males compared to isogenic controls; we thought a more dramatic change in lifespan would be observed if lifespan was measured under caloric restriction conditions.

Altering caloric content makes a significant difference in lifespan of wild type flies, so the lifespan of c754-m was measured on flies fed food of different caloric content (Pletcher et al., 2002). For measuring lifespan under caloric restriction, flies were raised on "standard" sucrose yeast media used by Pletcher et al. Flies enclosed for a 24-hour period. After eclosion, flies were placed over fresh "standard" sucrose yeast media for two days and then switched to one of the following foods of desired caloric content for the rest of their life: 0.65X sucrose yeast media, 1X (standard) sucrose yeast media, or 1.5X sucrose yeast media. The switch in nutrient concentration in the sucrose yeast media simulates caloric restriction. The 0.65x food has 35% less sucrose and yeast then a standard sucrose yeast media. 1.5X food has 50% more sucrose and yeast then a standard sucrose yeast media.
Figure 3.5 c754 females live significantly longer than the isogenic control females ($\Delta$c754-30) when fed cornmeal agar media. c754 (red) females or control (blue) females were kept at 25°C in demography cages at approximately 300 flies per cage. Every two days food was changed and dead flies were removed and counted. Results shown are the mean +/- S.E.M three separate demography cages. The red and blue arrows are the mean lifespan of c754 and control respectively. Unpaired Student T-test: test $t_4=3.03$; $p=0.04$. 
Figure 3.6 c754 males do not live significantly longer than the isogenic control males (Δc754-30) when fed cornmeal agar media. c754 (red) males or control (blue) males were kept at 25°C in demography cages at approximately 300 flies per cage. Every two days food was changed and dead flies were removed and counted. Results shown are the mean +/- S.E.M three separate demography cages. The red and blue arrows are the mean lifespan of c754 and control respectively. Unpaired Student T-test: test t₄=1.27; p=0.27.
The lifespan of c754-m is unaltered compared to Δc754-30 (control) when switched to lower nutrient food (0.65X sucrose yeast media). Females raised on 0.65X media have a mean lifespan of 47.6 +/- 2.29 days and 42.7 +/- 0.9 days for c754-m and Δc754-30 lines, respectively (fig. 3.7). Similarly, there is no change in lifespan in c754-m males and Δc754-30 males when raised on 0.65X sucrose yeast media. When raised on 0.65X media, c754-m males have a mean lifespan of 53.2 +/- 5.2 days compared to the Δc754-30 males with a mean lifespan of 55.0 +/- 2.5 days (fig. 3.8).

Keeping the flies on standard sucrose yeast media causes a slight decrease in female lifespan between c754-m and the Δc754-30 line but not in male lifespan (fig 3.9 and 3.10). c754-m females have a mean lifespan of 40.7 +/- 5.0 days compared to the Δc754-30 females with a mean lifespan of 51.9 +/- 3.3 days. In contrast, mean lifespan of c754-m males (50.2 +/- 1.1 days) is not different from Δc754-30 males (51.9 +/- 3.3 days) when raised on standard sucrose yeast media.

There is a significant change in lifespan when flies were switched to 1.5X sucrose yeast media. Mean lifespan of c754-m females (37.2 +/- 1.3 days) decrease as compared to Δc754-30 females (52.5 +/- 0.7 days) when raised on 1.5X sucrose yeast media (fig 3.11). Similar to the lifespan results of the females, c754-m males (44.3 +/- 2.0 days) have a decreased mean lifespan compared Δc754-30 males (57.8 +/- 2.3 days) (fig. 3.12).
Figure 3.7 *c754* females do not live significantly longer than the isogenic control females when fed 0.65X sucrose yeast agar media. *c754* (red) females or control (blue) females were kept at 25°C in demography cages at approximately 300 flies per cage. Every two days, food was changed and dead flies were removed and counted. The two life spans are equal when one adjusts for the aberrant data point on day 26. Results shown are the mean +/- S.E.M three separate demography cages. The red and blue arrows are the mean lifespan of *c754* and control respectively. Unpaired Student T-test: test *t* = 2.4; *p* = 0.07.
Figure 3.8 *c754* males do not live significantly longer than the isogenic control males when fed 0.65X sucrose yeast agar food. *c754* (red) males or control (blue) males were kept at 25°C in demography cages at approximately 300 flies per cage. Every two days, food was changed and dead flies were removed and counted. Results shown are the mean +/- S.E.M three separate demography cages. The red and blue arrows are the mean lifespan of *c754* and control respectively. Unpaired Student T-test: \( t_4 = 0.26; p = 0.8 \).
Figure 3.9 The lifespan of c754 females decreases compared to isogenic control females when fed 1X sucrose yeast agar media. c754 (red) females or control (blue) females were kept at 25°C in demography cages at approximately 300 flies per cage. Every two days, food was changed and dead flies were removed and counted. Results shown are the mean +/- S.E.M three separate demography cages. The red and blue arrows are the mean lifespan of c754 and control respectively. Unpaired Student T-test: test \( t_4 = 2.22; p = 0.09 \)
Figure 3.10 c754 males do not live significantly longer than the isogenic control when fed 1X sucrose yeast agar food. c754 (red) males or control (blue) males were kept at 25°C in demography cages at approximately 300 flies per cage. Every two days, food was changed and dead flies were removed and counted. Results shown are the mean +/- S.E.M three separate demography cages. The red and blue arrows are the mean lifespan of c754 and control respectively. Unpaired Student T-test: t₄=0.42; p=0.69.
Figure 3.11 c754 females have a significant decrease in lifespan compared to the isogenic control when fed 1.5X sucrose yeast food. c754 (red) females or control (blue) females were kept at 25°C in demography cages at approximately 300 flies per cage. Every two days, food was changed and dead flies were removed and counted. Results shown are the mean +/- S.E.M three separate demography cages. The red and blue arrows are the mean lifespan of c754 and control respectively. Unpaired Student T-test: test $t_4=10.1; p=0.0005$. 
Figure 3.12 c754 males have a significant decrease in lifespan compared to the isogenic control when fed 1.5X sucrose yeast media. c754 (red) males or control (blue) males were kept at 25°C in demography cages at approximately 300 flies per cage. Every two days, food was changed and dead flies were removed and counted. Results shown are the mean +/- S.E.M three separate demography cages. The red and blue arrows are the mean lifespan of c754 and control respectively. Unpaired Student T-test: t₄=4.4; p=0.01.
3.4 Molecular Analysis of c754

c754 has a P-element insertion that lies in the intronic region of the BR-C between the promoters and the translation start site (fig 3.2). This P-element insertion appears to be important for stress resistance as seen by the increase in starvation resistance compared to the isogenic control line. Preliminary work was done to look at BR-C expression levels using RT-PCR; there was no detectable change in expression levels of the BR-C transcripts. The more sensitive technique, quantitative real time RT-PCR (Q-PCR) (Bustin, 2000) was used to determine changes in BR-C expression levels of c754-m compared to the isogenic control, Δc754-30.

Q-PCR is a fluorescence based kinetic RT-PCR using either fluorophore nucleotide bound probes or the DNA binding dye Sybr Green for detecting amplification. PCR involves two phases of DNA amplification: the linear phase and the geometric phase. The linear phase is important in standard PCR. The geometric phase is important in Q-PCR. During the geometric phase, there is a two-fold increase in PCR product per cycle. Q-PCR uses the geometric phase to quantitate the amount of starting product because the amount of product made during the geometric phase is relative to the total amount of starting template. The total product made is measured by fluorescence at the end of each cycle, so that product production is measured in real time. Assays are compared at threshold fluorescence, which are fluorescence levels where all samples are in the geometric phase. During analyses, product amount is reported as a C_T value, which is the cycle number at which the product formation reaches the threshold value. Analysis of the gene expression by Q-PCR can be done by two methods: the standard curve method and the comparative C_T method (Applied Biosystems, 2001).
The standard curve method involves constructing either an absolute standard or a relative standard curve. The absolute curve is created by plotting the C_T values for known sample amounts at the same threshold level against the amount of the sample. The amount of gene expression in the experimental assay is determined by where the C_T values falls on the absolute standard curve. Since, this technique is limited in that to create an absolute curve, it is necessary to obtain sample dilutions in which the gene of interest represents a known percentage of the total sample. This limitation is overcome by using a relative standard curve, which involves constructing a curve from Q-PCR performed on a serial dilution of one sample where the gene of interest represents an unknown percentage of the total sample. The expression level of the sample is determined from the relative standard curve, and all samples are normalized to a common calibrator. The calibrator is commonly the control sample.

An alternative method is the comparative C_T method or ΔΔC_T method (Livak and Schmittgen, 2001). The comparative C_T method requires the efficiency of the gene of interest and the endogenous control to be the same. Fluorescence emission is graphed per cycle and the slope of this line during the geometric phase is dependent upon assay efficiency: equal slope implies equal efficiency of reaction. In this method, a ΔC_T is obtained for each template by subtracting the C_T of an endogenous control (housekeeping gene) from the C_T of the gene of interest. One template is chosen as a calibrator: the calibrator is usually the control sample. Relative expression amounts for all other templates are determined by first subtracting the ΔC_T of the calibrator from the ΔC_T of all templates to obtain a ΔΔC_T value. The natural log of -ΔΔC_T value is then calculated. This results in a relative expression value of one for the template chosen as the calibrator:
other templates are represented by expression values that are multiples or fractions of calibrator value.

I used the comparative $C_T$ method to analyze the Q-PCR results of $BR-C$ expression in c754 compared to the isogenic control. Five separate biological replicates of whole c754-m and Δc754-30 (control) females were examined for $BR-C$ expression levels. Males were not examined due to time constraints. The P-element insertion into the 5' UTR of the $BR-C$ of c754-m causes a decrease in expression of $BR-C$ transcripts in females. The relative expression of BRC-Z1 is 0.24 (0.18-0.32) in c754-m females compared to the Δc754-30 females that have a BRC-Z1 expression of 1 (0.85-1.18) (figs. 3.13 and 3.14). BRC-Z2 has little change in expression in c754-m, 0.65 (0.52-0.83), compared to Δc754-30, 1 (0.89-1.13), (figs. 3.15 and 3.16). BRC-Z3 expression decreases in c754-m females, 0.25 (0.18-0.35), relative to the Δc754-30 females, 1 (0.90-1.11) (fig 3.17 and 3.18). BRC-Z4 expression decreases from 1 (0.79-1.27) in Δc754-30 females to 0.32 (0.23-0.45) in c754-m females (figs 3.19 and 3.20).

The comparative $C_T$ method is not a valid method for analyzing the expression of the $BR-C$ isoforms in c754-m and the isogenic control, Δc754-30, because the assay efficiency of the $BR-C$ isoforms was not the same as the endogenous control, $RpL27$. To use the comparative $C_T$ method to analyze $BR-C$ expression, the $BR-C$ Q-PCR reaction needs to be optimized for better assay efficiency. Optimization can involve new primers or different starting template amount. An alternative would be to use the standard curve method for analyzing Q-PCR results to determine the expression levels of $BR-C$ in c754-m and Δc754-30.
Figure 3.13 Q-PCR amplification plot of BRC-Z1 expression in c754-m and isogenic control (Δc754-30) females. This plot is the amplification plot of BRC-Z1 amplification and Rpl27 (housekeeping gene) of one sample of five RNA sample for each genotype. The Y-axis is the measurement of fluorescence. The X-axis is the cycle at which the fluorescence is measured. The Rpl27 plot for each sample cannot be teased out suggesting each reaction had equal starting template. The green line identifies the threshold. This line can be adjusted to pick a threshold were there is equal efficiency in each reaction. Calculations are made from the cycle value at the threshold level, C_T.
Figure 3.14 Relative expression of BRC-Z1 in c754-m females compared to isogenic control females (Δc754-30) based on analysis of Q-PCR results. The comparative C_T method was used to analyze the Q-PCR results. c754-m has 0.24 the amount of BRC-Z1 expression compared to the isogenic control with a range of 0.18 to 0.32. The amount of BRC-Z1 expression if the isogenic control was used as the calibrator for the calculations and has a value of 1 with a range of the mean of 0.85 to 1.18. N=5 for each genotype.
Figure 3.15 Q-PCR amplification plot of BRC-Z2 expression in c754-m and isogenic control (Δc754-30) females. This plot is the amplification plot of BRC-Z2 amplification and RpL27 (housekeeping gene) of one sample of five RNA sample for each genotype. The Y-axis is the measurement of fluorescence. The X-axis is the cycle at which the fluorescence is measured. The RpL27 plot for each sample cannot be teased out suggesting each reaction had equal starting template. The green line identifies the threshold. This line can be adjusted to pick a threshold were there is equal efficiency in each reaction. Calculations are made from the cycle value at the threshold level, C_{T}. 
Figure 3.16 Relative expression of BRC-Z2 in c754-m females compared to isogenic control females (Δc754-30) based on analysis of Q-PCR results. The comparative $C_T$ method was used to analyze the Q-PCR results. c754-m has 0.65 the amount of BRC-Z2 expression compared to the isogenic control with a range of 0.50 to 0.83. The amount of BRC-Z2 expression of the isogenic control was used as the calibrator for the calculations and has a value of 1 with a range of 0.89 to 1.13. N=5 for each genotype.
Figure 3.17 Q-PCR amplification plot of BRC-Z3 expression in c754-m and isogenic control (Δc754-30) females. This plot is the amplification plot of BRC-Z3 amplification and Rpl27 (housekeeping gene) of one sample of five RNA sample for each genotype. The Y-axis is the measurement of fluorescence. The X-axis is the cycle at which the fluorescence is measured. The Rpl27 plot for each sample cannot be teased out suggesting each reaction had equal starting template. The green line identifies the threshold. This line can be adjusted to pick a threshold were there is equal efficiency in each reaction. Calculations are made from the cycle value at the threshold level, C_T.
Figure 3.18 Relative expression of BRC-Z3 in c754-m females compared to isogenic control females (Δc754-30) based on analysis of Q-PCR results. The comparative $C_T$ method was used to analyze the Q-PCR results. c754-m has 0.65 the amount of BRC-Z3 expression compared to the isogenic control with a range of 0.50 to 0.83. The amount of BRC-Z3 expression of the isogenic control was used as the calibrator for the calculations and has a value of 1 with a range of 0.89 to 1.13. N=5 for each genotype.
Figure 3.19 Q-PCR amplification plot of BRC-Z4 expression in c754-m and isogenic control (Δc754-30) females. This plot is the amplification plot of BRC-Z4 amplification and RpL27 (housekeeping gene) of one sample of five RNA sample for each genotype. The Y-axis is the measurement of fluorescence. The X-axis is the cycle at which the fluorescence is measured. The RpL27 plot for each sample cannot be teased out suggesting each reaction had equal starting template. The green line identifies the threshold. This line can be adjusted to pick a threshold were there is equal efficiency in each reaction. Calculations are made from the cycle value at the threshold level, $C_T$. 
Figure 3.20 Relative expression of BRC-Z4 in c754-m females compared to isogenic control females (Δc754-30) based on analysis of Q-PCR results. The comparative C_T method was used to analyze the Q-PCR results. c754-m has 0.32 the amount of BRC-Z4 expression compared to the isogenic control with a range of 0.23 to 0.45. The amount of BRC-Z4 expression of the isogenic control was used as the calibrator for the calculations and has a value of one with a range of 0.79 to 1.27. N=5 for each genotype.
3.5 Discussion of Results

The P-element insertion of c754 mutants alters BR-C expression and increases stress resistance and longevity compared to control flies supporting the notion that BR-C is involved in stress resistance and longevity. Both c754 males and females demonstrate an increase tolerance to starvation, yet only the females have an increase in lifespan under normal growth conditions (table 3.1). To further address the role of BR-C in stress response additional stress assays should be done. These stress assays would confirm the role of BR-C in stress response. The difference in longevity observed in c754 females but not males might be explained by c754 is a weak effector of BR-C expression and that the increase in male lifespan is not observed because measurements of lifespan are taken every other day instead of daily. The increase in lifespan and starvation survival in c754 females might not result from altered BR-C expression, but might be from a reduction in reproductive capabilities. Preliminary egg laying assays indicate that c754 lays fewer eggs than control females. Further testing of the fertility of c754 needs to be done to determine if c754 has reduced reproductive capabilities. If reproduction is found to be reduced this may suggest that the increase in starvation tolerance and lifespan of c754 females may be the result of a trade-off between longevity and reproductive abilities. To examine if the increase in starvation tolerance and lifespan in c754 females is correlated to decreased fertility or decreased expression of BR-C, the lifespan and starvation tolerance should be compared to the female sterile line, ovo^{D1}. If lifespan and starvation tolerance of c754 females is comparable to ovo^{D1} females, then one could speculate that the increase in lifespan and starvation tolerance of c754 females is caused by decreased fertility. If lifespan and starvation tolerance of c754 females is greater then that of the
<table>
<thead>
<tr>
<th>Line</th>
<th>Mean Life Span +/- S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c754-m ♀</td>
</tr>
<tr>
<td>Starvation</td>
<td>118 +/- 1.0 Hours</td>
</tr>
<tr>
<td>Cornmeal Agar</td>
<td>47.5 +/- 2.3 Days</td>
</tr>
<tr>
<td>0.65X Sucrose Yeast</td>
<td>46.8 +/- 2.3 Days</td>
</tr>
<tr>
<td>1X Sucrose Yeast</td>
<td>41.1 +/- 4.7 Days</td>
</tr>
<tr>
<td>1.5X Sucrose Yeast</td>
<td>38.4 +/- 0.8 Days</td>
</tr>
</tbody>
</table>

Table 3.1 Summary of c754 lifespan compared to control lifespan under different dietary conditions
ovo

females, then one could speculate that, the increase in lifespan and starvation
tolerance in c754 females is caused at least in part by the decrease of BR-C expression in
c754 females.

The lifespan results from the caloric restriction data suggest that the altered in
lifespan and starvation tolerance of flies raised on cornmeal agar food are not the result ofaltering the insulin-signaling pathway. One cannot directly compare lifespan of flies
raised on cornmeal agar to the lifespan of the flies in the caloric restriction experiments
because the sucrose yeast media in the caloric restriction experiments is very different
from the cornmeal agar media. Yeast and sucrose are the major ingredients in sucrose
yeast media whereas cornmeal and dextrose are the major ingredients in cornmeal agar
media. As other studies have found that lifespan decreases with increasing yeast or
nutrients (Sgro and Partridge, 1999; Pletcher et al., 2002), it was surprising to see no
decrease in lifespan of the isogenic control flies with increased nutrients. One might
argue that the control line is less capable of storing nutrients and therefore better food
quality allows it to live longer. Alternatively, the P-element of c754 might have
reinserted into an alternate place in the genome while silencing the w

marker. To
address the first concern, more precise excision lines should be generated for c754 and
tested for starvation tolerance and lifespan. Additionally, one can backcross the c754
mutant into several different wild type lines to create controls with similar genetic
backgrounds. To address the second concern, Southern analyses of the genomic DNA of
the control line should be done with probes designed to bind the P-element.

The Q-PCR data indicate that BR-C expression decreases in c754 females. This is
a good start for determining how c754 alters BR-C expression. For clearer data, the Q-
PCR for each of the BR-C isoforms needs to be optimized to use the comparative C_T method for analyses of gene expression. As mentioned in the description of Q-PCR, only assays of equal efficiency can be compared by the comparative C_T method. Efficiency can be grossly determined by examining the slope of the amplification plot during the geometric phase of amplification. Examination of the amplification plots of each BR-C isoform indicates that the Q-PCR efficiency of each isoform does not amplify as efficiently as the housekeeping gene. Trying new primer pairs, different template concentrations, or adjusting the Mg^{2+} concentration can improve efficiency of the Q-PCR reaction of each BR-C isoform. An alternative to the comparative C_T method would be to analyze the Q-PCR data by constructing a standard curve. After optimization of the Q-PCR reaction has occurred, then it would be wise to test c754 expression of BR-C isoforms in tissues rather then whole flies. Altered effects of BR-C expression in c754 may be more dramatic in specific tissues. It may be that BR-C expression is increased in the ovaries of the fly, but globally decreased.

One could try to examine the tissue where BR-C is expressed by using the intrinsic Gal4 expression of the c754 mutant. Based on the location of the c754 P-element within the BR-C, it would be expected that Gal4 would be expressed both temporally and spatially whenever BR-C is expressed within the C754 mutant. One can visualize the expression in the progeny of c754 crossed to either a UAS-GFP or UAS-LacZ transgenic flies. This expression pattern may illustrate BR-C expression in adult, but it will not determine how temporal and spatial expression of BR-C is altered in c754 mutants.
Chapter 4: Lifespan Increase by BR-C Overexpression is Isoform Dependent

The stress resistant mutant, c754 shows decreased expression of three BR-C isoforms. Comparison of BR-C overexpression in transgenic lines to non-transgenic controls will help determine if lower BR-C expression is the cause of stress resistance. Transgenic flies with expression of one of the BR-C isoforms (BRC-Z1, BRC-Z2, BRC-Z3, or BRC-Z4) were tested for survival during starvation. The overexpression of BRC-Z1, BRC-Z2, and BRC-Z4 isoforms resulted in increased survival time in the absence of food. In chapter four, this work will be complemented by analysis of lines with decreased BR-C expression.

4.1 Generating Overexpression Transgenic lines

To overexpress BR-C isoforms, transgenic fly lines were created in which the Hsp70 promoter was used to drive expression of each isoform. The hsp70 promoter induces ubiquitous expression of a given gene when exposed to elevated temperatures. Four transgenic fly lines carrying heatshock inducible BR-C (HS-BRC) isoforms were obtained from DR. Cynthia Bayer (Bayer et al., 1997). Crossgrove et al. and Bayer et al tested the ability of these lines to express the desired BR-C isoform two ways: first by western blot analysis of BR-C isoform expression in BR-C null mutants and rescue of larval lethal BR-C mutants (Bayer et al., 1997; Crossgrove et al., 1996). I then tested these confirmed HS-BR-C transgenic lines for starvation resistance compared to non-transgenic lines.
To carefully compare the starvation resistance of the transgenic lines to a non-transgenic control, I derived control lines with the same genetic background as the transgenic line. Homozygous transgenic fly lines were crossed with their progenitor line. The progeny from this cross were allowed to mate, giving rise to progeny that will segregate for the transgene in a 1:2:1 ratio. Any background sensitivity to starvation in either the original transgenic line or the wild type line would have an equal chance of segregating in these crosses, thus creating a transgenic line and a control line in the same randomized genetic background. Homozygous, heterozygous, and control (non-transgenic) siblings were separated from each other based on eye color. Specifically, the selectable marker for the transgene is \( w^+ \), which results in red eyes in a \( w^- \) background. Homozygous transgenic progeny will have eyes that are a darker shade of red than heterozygous transgenic progeny because it carries two copies of the \( w^+ \) gene. The control (non-transgenic) progeny have white eyes.

For starvation testing, \( BR-C \) transgenic flies and non-transgenic flies were separately heatshocked at 37°C for one hour on normal food. Eight hours after the initial heatshock, flies were switched to vials containing 1% agar only and heatshocked every 24 hours for one hour to maintain transgene expression levels.

4.2 Overexpression of BRC-Z1 Increases Starvation Tolerance

Analyses of mean starvation survival time of females indicate that homozygous HS-BRC-Z1 flies (103.3+/-.3.5 hours) and heterozygous HS-BRC-Z1 flies (102.5 +/- 1.9 hours) have a significant increase in starvation tolerance compared to control flies (91.6 +/- 0.8 hours) (fig. 4.1). There is no significant difference in starvation tolerance between
Figure 4.1 The increased starvation survival by overexpression of BRC-Z1 is dominant in females. Homozygous HS-BRC-Z1 (Yellow), heterozygous HS-BRC-Z1 (Green) or control (+) (blue) line were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. For one hour daily, flies were heatshocked in a 37°C incubator. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The blue, green, and yellow arrow mark the mean lifespan for the control, heterozygous, and homozygous lines, respectively. Unpaired Student’s T-test values for homozygous line compared to control line: t₄ = 3.3; p=0.03. Unpaired Student’s T-test values for heterozygous line compared to control line: t₄ = 5.3; p=0.006.
the homozygous and heterozygous HS-BRC-Z1 females. A similar increase in
starvation resistance is observed in HS-BRC-Z1 males. The mean starvation time of
homozygous HS-BRC-Z1 males (95 +/- 2.9 hours) is increased significantly compared to
heterozygous HS-BRC-Z1 males (81.8 +/- 3.2 hours) and control males (78.7 +/- 1.5
hours) (fig 4.2). There is no significant increase in starvation survival between the
heterozygous HS-BRC-Z1 male and control males. BRC-Z1 overexpression increases
starvation tolerance. Interestingly, the overexpression of BRC-Z1 in females has a
dominant effect on increasing starvation survival, while the overexpression of BRC-Z1 in
males has a dosage dependent effect on increasing starvation tolerance.

4.3 Overexpression of BRC-Z2 Increases Starvation Tolerance

To determine the specificity of BR-C effect on starvation tolerance additional BR-
C isoforms were tested. The mean starvation survival time for homozygous HS-BRC-Z2
females (119.3 +/- 6.4 hours) and the heterozygous HS-BRC-Z2 females (109.5 +/- 3.1
hours) are greater than the mean starvation survival time of control females (100.1 +/- 2.2
hours) (fig 4.3). The increase in starvation of the homozygous HS-BRC-Z1 females
compared to control females is significant. Transgenic males show a similar effect on
starvation survival. The mean starvation survival of the homozygous HS-BRC-Z2 males
(112.2 +/- 3.0 hours) is significantly greater than both the mean starvation survival time
of the heterozygous HS-BRC-Z2 males (95.8 +/- 4.3 hours) and control males (82.2 +/-
2.8 hours) (fig. 4.4).
Figure 4.2 The increased starvation survival by overexpression of BRC-Z1 is dosage dependent in males. Homozygous HS-BRC-Z1 (Yellow), heterozygous HS-BRC-Z1 (Green) or control (+) (blue) line were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. For one hour daily, flies were heatshocked in a 37°C incubator. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The blue, green, and yellow arrow mark the mean lifespan for the control, heterozygous, and homozygous lines, respectively. Unpaired Student’s T-test values for homozygous line compared to control line: t₄ = 5.1; p=0.007. Unpaired Student’s T-test values for heterozygous line compared to control line: t₄ = 0.9; p=0.4.
Figure 4.3 The increased starvation survival by overexpression of BRC-Z2 is dosage dependent in females. Homozygous HS-BRC-Z2 (Yellow), heterozygous HS-BRC-Z2 (Green) or control (+) (blue) line were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. For one hour daily, flies were heatshocked in a 37°C incubator. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The blue, green, and yellow arrow mark the mean lifespan for the control, heterozygous, and homozygous lines, respectively. Unpaired Student’s T-test values for homozygous line compared to control line: t₄ = 2.8; p=0.04. Unpaired Student’s T-test values for heterozygous line compared to control line: t₄ = 2.4; p=0.07.
Figure 4.4 The increased starvation survival by overexpression of BRC-Z2 is dosage dependent in males. Homozygous HS-BRC-Z2 (Yellow), heterozygous HS-BRC-Z2 (Green) or control (+) (blue) line were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. For one hour daily, flies placed in a 37°C incubator. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The blue, green, and yellow arrow mark the mean lifespan for the control, heterozygous, and homozygous lines, respectively. Unpaired Student’s T-test values for homozygous line compared to control line: t₄ = 6.9; p=0.002. Unpaired Student’s T-test values for heterozygous line compared to control line: t₄ = 2.3; p=0.06.
4.4 Overexpression of HS-BRC-Z3 Does Not Change Starvation Tolerance

There is no significant change in mean lifespan of homozygous or heterozygous HS-BRC-Z3 transgenic flies compared to control flies. Homozygous and heterozygous HS-BRC-Z3 females have a mean starvation lifespan of 109.3 +/- 0.9 hours and 107.3 +/- 0.9 hours respectively; Control females have a mean starvation survival time of 106.7 +/- 2.3 hours (fig 4.5). The same result is observed in males. Homozygous and heterozygous HS-BRC-Z3 males have a mean starvation lifespan of 92.8 +/- 4.5 hours and 89.2 +/- 2.2 hours respectively; Control males have a mean starvation lifespan of 92.0 +/- 1.45 hours (fig 4.6).

4.5 Overexpression of HS-BRC-Z4 Increases Starvation Tolerance

BRC-Z4 is considered a weak form of BRC-Z1 and has been found to partially compensate loss of BRC-Z1 function. It is expected that overexpression of BRC-Z4 would result in a similar starvation tolerance as the BRC-Z1 overexpressing line. The mean starvation lifespan of both homozygous HS-BRC-Z4 females (121.0 +/- 5.3 hours) and heterozygous HS-BRC-Z4 females (114.8 +/- 4.0 hours) is significantly greater than the mean starvation lifespan of the control females (104.3 +/- 2.3 hours) (fig. 4.7). However, heterozygous HS-BRC-Z4 females do not have a significant change in starvation survival compared to the homozygous HS-BRC-Z4 females. Transgenic males show a similar starvation survival phenotype to transgenic females. The mean starvation lifespan of homozygous HS-BRC-Z4 (100.7 +/- 2.4 hours) is significantly greater than the mean starvation lifespans of heterozygous HS-BRC-Z4 (89.0 +/- 2.6 hours) and control males (85.0 +/- 0.6 hours) (fig 4.8).
Figure 4.5 Overexpression of BRC-Z3 does not alter starvation tolerance in females. Homozygous HS-BRC-Z3 (Yellow), heterozygous HS-BRC-Z3 (Green) or control (+) (blue) line were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. For one hour daily, flies were heatshocked in a 37°C incubator. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line.
Figure 4.6 Overexpression of BRC-Z3 does not alter starvation tolerance in males. Homozygous HS-BRC-Z3 (Yellow), heterozygous HS-BRC-Z3 (Green) or control (+) (blue) line were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. For one hour daily, flies were placed in a 37°C incubator. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line.
Figure 4.7 The increased starvation survival by overexpression of BRC-Z4 is dominant in females. Homozygous HS-BRC-Z4 (Yellow), heterozygous HS-BRC-Z4 (Green) or control (+) (blue) line were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. For one hour daily, flies were heatshocked in a 37°C incubator. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The blue, green, and yellow arrow mark the mean lifespan for the control, heterozygous, and homozygous lines, respectively. Unpaired Student’s T-test values for homozygous line compared to control line: t_4 = 2.9; p=0.04. Unpaired Student’s T-test values for heterozygous line compared to control line: t_4 = 2.3; p=0.08.
Figure 4.8 The increased starvation survival by overexpression of BRC-Z4 is dosage dependent in males. Homozygous HS-BRC-Z4 (Yellow), heterozygous HS-BRC-Z4 (Green) or control (+) (blue) line were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. For one hour daily, flies were heatshocked in a 37°C incubator. Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The blue, green, and yellow arrow mark the mean lifespan for the control, heterozygous, and homozygous lines, respectively. Unpaired Student’s T-test values for homozygous line compared to control line: $t_4 = 6.3; p=0.003$. Unpaired Student’s T-test values for heterozygous line compared to control line: $t_4 = 1.5; p=0.2$. 
4.6 Discussion

Heatshock induced overexpression of BRC-Z1, BRC-Z2, and BRC-Z4 results in increased starvation resistance compared to controls strengthening the argument that BR-C influences stress response (table 4.1). These results are encouraging, but one needs to confirm that the desired BR-C isoform after heatshock induction is being overexpressed, that this overexpression is global, and the amount of BR-C expression over the time course of the starvation assay.

I assumed that upon heatshock the amount of the desired HS-BRC isoform expression increases. I confirmed that 30 minutes after heatshock, HS-BRC-Z1 is overexpressed compared to its control with no detectable change in expression of the other BR-C isoforms. The amount of overexpression per tissue in the HS-BRC lines remains unclear. It is assumed that overexpression is global, but the expression of the transgene is highly dependent on the insertion site of the transgene in the genome. The region of the chromosome in which the transgene inserted may be more readily transcribed in one tissue that another tissue. Global expression can be determined by assaying the RNA levels of the HS-BRC transgene in various tissues and body parts, 30 minutes after heatshock of the transgenic flies. Additionally, mRNA levels of the desired BR-C isoform should increase after heatshock, but the mRNA level decreases over time until the next heatshock. It remains unclear how the fluctuation of BR-C levels influences starvation resistance. The fluctuation in BR-C mRNA levels can be examined by measuring the RNA levels at several time points after heatshock.
| # of Transgenes Transgene | Females | | | Males | | | |
|---|---|---|---|---|---|---|
| | Homozygous | Heterozygous | Control | Homozygous | Heterozygous | Control | |
| Hs-BRC-Z1 | 103.3 +/- 3.5 Hours | 102.5 +/- 1.9 Hours | 91.6 +/- 0.8 Hours | 95.0 +/- 2.9 Hours | 81.8 +/- 3.2 Hours | 78.7 +/- 1.5 Hours | |
| Hs-BRC-Z2 | 119.0 +/- 6.4 Hours | 109.5 +/- 3.1 Hours | 100.1 +/- 2.2 Hours | 112.2 +/- 3.0 Hours | 95.8 +/- 4.3 Hours | 82.2 +/- 2.8 Hours | |
| Hs-BRC-Z3 | 109.3 +/- 0.9 Hours | 107.3 +/- 0.9 Hours | 106.7 +/- 2.3 Hours | 92.8 +/- 4.5 Hours | 89.2 +/- 2.2 Hours | 92.0 +/- 1.5 Hours | |
| Hs-BRC-Z4 | 121.0 +/- 5.3 Hours | 114.8 +/- 4.0 Hours | 104.3 +/- 2.3 Hours | 100.7 +/- 2.6 Hours | 89.0 +/- 2.6 Hours | 85.0 +/- 0.6 Hours | |

Table 4.1 Summary of mean lifespan under starvation conditions of HS-BRC transgenic lines compared to control.
The increased starvation tolerance of three of the four BR-C overexpressing lines conflicts with the c754 increase in starvation tolerance with decreases in BR-C expression. The conflicting outcome of both overexpression and underexpression of BR-C resulting in increased lifespan may be a result of BR-C regulation or a consequence of decreased fertility. It is conceivable that overexpression of one BR-C isoform causes the downregulation of another BR-C isoform. Overexpression of the BRC-Z1, BRC-Z2, and BRC-Z4 isoforms may yield increased starvation survival by decreasing expression of the other BR-C isoforms. To examine this idea, expression levels of all BR-C isoforms should be examined at various times points after heatshock in all the BR-C overexpressing lines. The expression levels of the BR-C isoforms in the overexpressing lines should then be compared to the BR-C isoform expression levels of c754. The BR-C isoform that is responsible for the increase in starvation tolerance should be downregulated equally in the c754 mutant and the overexpressing lines.

Another explanation for the overexpression of BR-C to have a similar starvation phenotype as c754, is that the overexpressing lines have delayed eclosion and defective reproduction suggesting that the increase in starvation tolerance could be the result of resources being conserved instead of being used for reproduction. This can be examined by testing the fecundity and eclosion rate of the homozygous transgenic flies, heterozygous transgenic flies, and control flies. If there were a decline in reproduction, it would support the theory that starvation tolerance is caused by the decrease in reproductive capabilities. If decreased reproduction is an underlying factor of starvation survival when BR-C expression is increased, a double mutant of the HS-BRC transgenics and the female sterile ovoD1 should be generated. ovoD1 lacks complete reproductive
abilities in females. The effect of reproduction would be irrelevant in the starvation survival of the double mutant; therefore establishing if misexpression of $BR-C$ does or does not increase starvation survival.

Interestingly, ubiquitous overexpression of BRC-Z1 has a dominant effect in females and a dosage effect in males. It is plausible that there is a threshold level of $BR-C$ expression that once reached no greater amount of starvation survival can be obtained. One could argue that females have a lower $BR-C$ expression threshold than males therefore the heterozygous transgenic expression levels reach the threshold maximum in females, but not in males. Females may have a lower threshold level of $BR-C$ because of the role of $BR-C$ in oogenesis. It has been demonstrated that the overexpression and underexpression of $BR-C$ causes defects in oogenesis (Deng and Bownes, 1997; Tzolovsky et al., 1999). Females may have more checkpoints and regulation of $BR-C$ expression because of the consequences of altered $BR-C$ expression has on oogenesis. No studies of $BR-C$ function in spermatogenesis has been done. One could examine this threshold theory by comparing the BR-C expression levels in wild type males and females. In addition, one can test starvation survival in males with more copies of the transgene to try to reach the threshold level of $BR-C$ expression where more $BR-C$ does not increase starvation survival further.
Chapter 5: Underexpression of BR-C isoforms using RNAi

An additional genetic approach to establish the role of BR-C in stress response is to decrease BR-C expression levels by RNA interference (RNAi). If decreased BR-C expression as seen in c754 were important for increased starvation resistance, then it would be expected that RNAi silencing of BR-C would cause flies to live longer under starvation conditions. However, overexpression of BR-C results in an increase in stress resistance as well. RNAi is a useful tool that may resolve the conflicting starvation survival data of c754 and BR-C overexpression.

5.1 Background on RNAi

RNA interference (RNAi) is a relatively new technique used to lower RNA expression levels by silencing RNA at the post-transcriptional level by recognition of the mRNA by targeted double-stranded RNA (dsRNA) (Hammond et al., 2001). The protein dicer is a ribonuclease that makes small interfering RNA (siRNA) by specifically cleaving dsRNA into 22bp fragments (fig 5.1) (Bernstein et al., 2001; Dillin, 2003; Ketting et al., 2001). Dicer is a highly conserved protein with homologs found in the plant, animal, and fungal kingdoms. siRNA acts as a guide RNA forming a complex with proteins to degrade target mRNAs. RNAi can be expressed in Drosophila in a tissue specific manner.

For tissue specific expression of RNAi in Drosophila, the Gal4/UAS system is employed (Brand, A. H., and Perrimon, N., 1993). Briefly, the Gal4/UAS system employs the yeast transcription factor Gal4 which recognizes a sequence called the
Figure 5.1 Overview of RNAi-mediated mRNA degradation. On entry into cells, dsRNA is cleaved by Dicer into 21- to 23-nt siRNAs. siRNAs are complexed with a large multi-protein complex, the RISC. RISC is thought to unwind the siRNA to help target the appropriate mRNA (shown in green). The siRNA/mRNA hybrid is cleaved, releasing the siRNA, and the mRNA is degraded by endo and exonucleases. Dillin, Andrew (2003) Proc. Natl. Acad. Sci. USA 100, 6289-6291
Upstream Activating Sequence (UAS). In *Drosophila*, the coding sequence for Gal4 is placed under control of a tissue specific promoter allowing Gal4 to be expressed temporally and spatially within the tissue of interest. The UAS sequence can be placed in front of reporter genes, endogenous genes or genes that can down regulate function or expression via dominant negative mutants or RNAi. Progeny of flies from Gal4 transgenic flies mated to flies with a UAS transgene will express the UAS transgene when the promoter upstream of the Gal4 is activated.

There are two ways to employ UAS expressed RNAi in *Drosophila*: either by using an inverted repeat of part of the gene of interest to express a dsRNA that forms by annealing back by a hairpin loop or by simultaneous expression of both a sense and antisense RNA (Billuart et al., 2001, Giordano et al., 2002, Fortier and Belote, 2000; Kalidas and Smith, 2002; Kennerdell and Carthew, 2000; Lam and Thummel, 2000; Martinek and Young, 2000; Piccin et al., 2001). Dual transcription of sense and antisense RNA is done by placing UAS sequences on either side of the fragment of the gene of interest. To facilitate this concurrent expression of sense and antisense expression, the pSymp-UAST plasmid was created (Giordano et al., 2002). Simultaneous transcription of sense and antisense was preferred over inverted repeats because inverted repeats are mitotically unstable and stimulate genomic rearrangements in eukaryotic cells (Giordano et al., 2002).

RNAi transgenic flies were made with flies that contain the pSymp-UAST vector driving expression of a *BR-C* fragment that is specific towards that desired *BR-C* isoform: BRC-core, BRC-Z1, BRC-Z2, BRC-Z3, and BRC-Z4. The *BR-C* isoforms have high sequence similarities; therefore, it was crucial to choose a portion of the sequence for
each isoform that would be specific to that isoform. The optimal length of the target fragment to be cloned into the RNAi vectors is approximately 500bp. The fragments selected for the RNAi of the BR-C isoforms were 150bp to 550 bp. The fragment chosen was the most specific to the isoform with no cross hybridization of the fragment to other isoforms (Bayer et al., 1996).

5.2 Driving Expression of the RNAi Construct Using Act-5 Gal4

To determine the ability of the RNAi construct to reduce or eliminate levels of BR-C, the RNAi transgenic flies were crossed to flies containing the Act-5 Gal4 transgene. The Act-5 Gal4 transgene drives expression of Gal4 where actin is expressed which is virtually everywhere and at all times. Act-5 Gal4 is considered one of the strongest Gal4 drivers and is a good start to determine the effectiveness of the RNAi constructs.

It was expected that if the RNAi constructs reduced BR-C expression when expressed by the Act-5 Gal4 driver that there would be a morphological phenotype with defects generated the larval to adult transition during pupation. BR-C null mutants and BR-C mutants such as br$^*$, rbp$^l$, and 2bc$^l$, have lethal phenotypes at the pupal stage of development (Belyaeva et al., 1982; Emery et al., 1994; Karim et al., 1993; Kiss et al., 1988). It was expected that RNAi directed towards the core domain would behave as a BR-C null mutant, because it is active against all isoforms. Null mutants remain as wandering third instar larvae (Belyaeva et al., 1982; Emery et al., 1994). The other RNAi constructs should behave as the other lethal mutants that pupate but die either before full metamorphosis occurs or as pharate adults.
5.2.1 Act-5 Gal4 RNAi Directed Towards the Core Domain Results in Pupal Arrest

Flies heterozygous for the core RNAi transgene or the Act-5 Gal4 transgene carried over a CyO balancer were crossed. According to Mendelian genetics, 33% of the progeny from this cross are expected to have both the RNAi core transgene and the Act-5 Gal4 driver. No adults emerge from this cross, that contains both transgenes, but necrotic pupae were observed (fig 5.2). The necrotic pupae make up 29% of the progeny population and are thought to be the double transgenic progeny because they occur close in numbers to the expected value for the double transgenic progeny (fig 5.2). This suggests that RNAi core expression may reduce all BR-C expression levels but it does not eliminate BR-C expression, since the wandering third instar larval phenotype of null mutants is not observed (Belyaeva et al., 1982; Emery et al., 1994).

5.2.2 Act-5 Gal4 RNAi Directed Towards the BRC-Z1 Isoform Blocks Development at the Pharate Adult Stage

Flies homozygous for the BRC-Z1 RNAi transgene were crossed to flies with the Act-5 Gal4 driver carried over a CyO balancer. Progeny with both the BRC-Z1 RNAi transgene and the Act-5 Gal4 driver are expected to make up 50% of the progeny population. Pharate adults comprised 67% of the progeny population (fig 5.3). Based on phenotype of the progeny that eclosed, the pharate adults were the progeny that had both the Act-5 Gal4 driver and the BRC-Z1 RNAi transgene. The pharate adult phenotype is similar to rbp (Emery et al., 1994) indicating that Act-5 Gal4 expression of RNAi BRC-Z1 efficiently decreases BRC-Z1 expression.
Figure 5.2 Act-5 Gal4 expression of RNAi targeting the BRC-core domain results in pupal arrest. A) The cross of core RNAi virgin females with Act5-Gal4 males. B) The progeny from the cross (top row), the number of progeny and the percentage of progeny. The progeny observed are in agreement with Mendelian ratios.
A) 

<table>
<thead>
<tr>
<th>Cross:</th>
<th>w; Z1RNAi ; + (♀)</th>
<th>X</th>
<th>w; CyO/Act5-Gal4; + (♂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype of progeny</td>
<td>w; CyO/Z1RNAi ; +</td>
<td></td>
<td>w; Z1RNAi/Act5-Gal4; +</td>
</tr>
<tr>
<td>Expected frequency</td>
<td>50%</td>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>Observed numbers</td>
<td>159</td>
<td></td>
<td>131- pharate adult</td>
</tr>
<tr>
<td>Observed frequency</td>
<td>33%</td>
<td></td>
<td>67%</td>
</tr>
<tr>
<td>Eye color</td>
<td>orange</td>
<td></td>
<td>red orange</td>
</tr>
</tbody>
</table>

B) 

Figure 5.3 Act-5 Gal4 expression of RNAi targeting BRC-Z1 results in pharate adults.  
A) The BRC-Z1 RNAi cross to Act5-Gal4 driver with expected progeny for each genotype and actual progeny for each genotype’s) Pupa of progeny from both genotypes.  B-1) Pupa with both the Act5-Gal4 driver and pSympUAS-Z1 as determined by the red eye color.  The adult fly does not eclose from the pupal case.  Also, notice that the abdomen does not fully close (red arrow).  B-2) Sibling of B-1 but only possessing the RNAi Z1 no Gal4 as determined by the orange eyes.  Pupae with just the RNAi construct do eclose.
5.2.3 Act-5 Gal4 RNAi Directed Towards the BRC-Z2 Isoform Results in Pupal Arrest

Homozygous RNAi BRC-Z2 transgenic flies were crossed to Act-5 Gal4 flies over a CyO balancer to drive expression of the RNAi BRC-Z2 construct in progeny. 50% of the progeny are expected to have both transgenes. Progeny with both transgenes did not emerge, but necrotic pupae, as seen with RNAi core expression, were apparent (fig 5.4). The necrotic pupae comprise 41% of the pupal population, suggesting that the necrotic pupae are the double transgenic. BRC-Z2 is thought to be responsible for \( br^+ \) function. Lethal \( br \) mutants die prior to metamorphosis in the pupal case (Belyaeva et al., 1982; Karim et al., 1993). The pupal death of the double transgenic is similar to the lethal phenotype of the \( br \) mutants indicating that RNAi BRC-Z2 is decreasing BRC-Z2 expression.

5.2.4 Act-5 Gal4 RNAi Directed Against the BRC-Z3 Isoform Requires Two Copies of The RNAi BRC-Z3 Transgene to Observe a Pupal Arrest Phenotype

Flies that are transgenic for RNAi BRC-Z3 on either the second or the third chromosome were crossed to Act-5 Gal4 flies. RNAi BRC-Z3 on the second chromosome is not homozygous, while RNAi BRC-Z3 on the third is homozygous. The double transgenic progeny from the cross of Act-5 Gal4 to RNAi BRC-Z3 on the second chromosome are expected to comprise 25% of the total progeny. 50 % of the progeny from the cross of RNAi BRC-Z3 on the third chromosome to Act-5 Gal4 is expected to have both transgenes. Based on eye and wing phenotypes, double transgenic progeny
A)

<table>
<thead>
<tr>
<th>Cross:</th>
<th>w; Z2RI-1A (♀) × w; CyO/Act5-Gal4 (♂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype of progeny</td>
<td>w; CyO/Z2RI-1A; + w; Z2RI-1A/Act5-Gal4; +</td>
</tr>
<tr>
<td>Expected frequency</td>
<td>50% 50%</td>
</tr>
<tr>
<td>Observed numbers</td>
<td>189 129- all aberrant pupa, no eclosion</td>
</tr>
<tr>
<td>Observed frequency</td>
<td>59% 41%</td>
</tr>
<tr>
<td>Eye colors</td>
<td>orange not applicable</td>
</tr>
</tbody>
</table>

B)

Figure 5.4 Act-5 Gal4 expression of RNAi targeting BRC-Z2 results in pupal arrest. A) Cross of Z2RNAi to Act-5 Gal4 driver with expected progeny for each genotype and actual progeny for each genotype. B) Pupal phenotype for both genotypes. B-1) Z2 RNAi with no Gal4 expression. B-2) Z2 RNAi with Gal4 expression becomes necrotic and dies in the pupal case.
eclosed in the expected ratio. Unlike the other RNAi BR-C transgenes tested, RNAi targeting BRC-Z3 does not cause a pupal arrest phenotype. A pupal arrest phenotype was expected for RNAi targeting BRC-Z3 because $2bc^l$ flies die in the pupal stage of development (Karim et al., 1993; Kiss et al., 1980), and $2bc^l$ flies are supposedly defective in BRC-Z3 function (Bayer et al., 1997). Because RNAi is not 100% efficient, it is thought that BRC-Z3 RNA is reduced but not enough to prevent metamorphosis. To determine if BRC-Z3 expression was reduced, reverse transcriptase PCR (RT-PCR) was done on adults that are double transgenic for both the BRC-Z3 RNAi transgene and the Act-5 Gal4 driver. RT-PCR results of the double transgenic flies indicate that there is no detectable change in BRC-Z3 expression compared to single transgenic siblings (fig 5.5). BRC-Z3 RNAi may be dosage dependent; therefore, crosses were made to generate flies that carried multiple copies of the BRC-Z3 RNAi transgene when crossed to Act-5 Gal4. Flies that had two copies of the BRC-Z3 RNAi transgene and one copy of the Act-5 Gal4 driver pupate but die in the pupal case before morphogenesis occurs. This suggests that the BRC-Z3 RNAi efficiency is dosage dependent.

5.2.5 Act-5 Gal4 RNAi Directed Towards the BRC-Z4 Isoform Results in Partially Eclosed Adults

Homozygous BRC-Z4 RNAi transgenic flies were crossed to heterozygous Act-5 Gal4 carried over CyO balancer. Double transgenic progeny from the RNAi BRC-Z4 cross to Act-5 Gal4 are expected to comprise 50% of the total progeny. 51% of the progeny have the appropriate phenotype for both the RNAi BRC-Z4 transgene and the Act-5 Gal4 driver (fig 5.6). The double transgenic flies die either during eclosion or
Figure 5.5 Act-5 Gal4 expression of RNAi targeting BRC-Z3 does not decrease BRC-Z3 mRNA levels. RT-PCR of mRNA from flies either expressing RNAi Z3 or not expressing RNAi Z3 determined that there was not an observable decrease in Z3 expression when RNAi Z3 was active. Lane 1 is Act-5 Gal4 with RNAi Z3 on the third chromosome. Lane 2 is a sibling of lane 1, but with no Act-5 Gal4. Lane 3 is Act-5 Gal4 with RNAi Z3 on the second chromosome. Lane 4 and 5 are siblings of lane 3 missing either the Act-5 Gal4 driver or the RNAi Z3 construct. RpL27 is a housekeeping gene and is used as a load control.
A)

<table>
<thead>
<tr>
<th>Cross:</th>
<th>w; Z4 RNAi (♀) X w; CyO/Act5-Gal4 (♂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype of progeny</td>
<td>w; CyO/Z4RNAi ; + w; Z4RNAi/Act5-Gal4; +</td>
</tr>
<tr>
<td>Expected frequency</td>
<td>50% 50%</td>
</tr>
<tr>
<td>Observed numbers</td>
<td>154 160- partial eclosion, (8 eclosions)</td>
</tr>
<tr>
<td>Observed frequency</td>
<td>49% 51%</td>
</tr>
<tr>
<td>Parental eye colors</td>
<td>orange red orange</td>
</tr>
</tbody>
</table>

B)

Figure 5.6 Act-5 Gal4 expression of RNAi targeting BRC-Z4 results in partially eclosed adults. A) Cross of RNAiZ4 to Act-5 Gal4 with expected progeny for each genotype and actual progeny for each genotype. B) Expression of Z4 RNAi with Act5-Gal4 resulted in partially eclosed adults. Adults that were able to eclose did not survive longer than 24 hours.
shortly after eclosion (fig. 5.6). This is a less severe phenotype than the BRC-Z1 RNAi and the rbp mutant. This weaker lethal phenotype of BRC-Z4 RNAi corroborates the idea that the BRC-Z4 is a weak form of BRC-Z1.

5.3 Driving RNAi expression with and inducible Gal4 expression

If the c754 mutation enhanced starvation survival by underexpressing BR-C, then it was expected that underexpression of BR-C isoforms by RNAi should likewise enhance the starvation survival. The morphogenic lethality of Act-5 Gal4 driven expression of the RNAi constructs suggests that the RNAi constructs do lower the levels of the various isoforms. The interest of this thesis is to study the effect of the BR-C on stress response in adults; therefore, an inducible Gal4 driver must be used to obtain the adults that express RNAi targeting BR-C expression. The Gal4 driver chosen was a heatshock inducible Gal4 driver, which contains the heatshock 70 promoter in front of the Gal4 coding sequence. When heatshock Gal4 (HS-Gal4) flies are placed at 37°C, they express Gal4 in all tissues. Starvation resistance of double transgenic flies was compared to control flies containing only the RNAi transgene. Both the double transgenic flies and the single transgenic control flies were either exposed to a one-hour daily heatshock or kept at constant temperatures under starvation conditions.

5.3.1 Flies expressing RNAi BRC-Core Did Not Have a Change in Starvation Resistance.

Heatshock induction of BRC-core RNAi does not change starvation survival. Double transgenic females have a mean starvation lifespan if 100.7 +/- 5.7 hours and 108.0 +/- 1.7 hours when heatshocked for one hour daily or kept at constant temperature
respectively (fig 5.7). Similar mean starvation lifespans are observed in the single transgenic BRC-core RNAi females with daily heatshock (111.8 +/- 2.1 hours) and without daily heatshock (106.7 +/- 2.8 hours). As seen with females expressing RNAi targeting the core domain, males do not have a change in starvation resistance. Males possessing both the HS-Gal4 driver and the core RNAi transgene have a mean starvation resistance of 87.7 +/- 3.8 hours and 88.8 +/- 5.3 hours with and without daily heatshock, respectively (fig. 5.8). Single transgenic males that had only the core RNAi transgene have a mean lifespan under starvation of 85.0 +/- 2.5 hours and 90.6 +/- 4.7 hours with and without heatshock, respectively. Expression of BRC-core RNAi with the HS-Gal4 driver does not change starvation survival.

### 5.3.2 Females Expressing RNAi BRC-Z1 Have a Significant Decrease in Starvation Lifespan

Females that express RNAi BRC-Z1 have a significant decrease in lifespan under starvation, but males expressing RNAi BRC-Z1 do not. Heatshocking females with both the HS-Gal4 transgene and the BRC-Z1 RNAi transgene resulted in a decrease in mean starvation survival (91.0 +/- 0.2 hours) compared to the mean starvation time of double transgenic females (96.6 +/- 1.0 hours) that remain at constant temperatures and heatshocked RNAi BRC-Z1 transgenic females (99.9 +/- 2.9 hours) (fig 5.9). Single transgenic females kept at room temperature have a comparable starvation resistance (106.4 +/- 2.4 hours) to the double transgenic kept at constant temperature. In contrast, heatshocking males with both the HS-Gal4 and RNAi BRC-Z1 transgenes does not alter starvation survival (fig. 5.10).
Figure 5.7 Females expressing RNAi directed towards the core domain do not have a change in starvation resistance compared to females not expressing core RNAi. Flies with the core RNAi construct were mated to either HS-Gal4 flies or w^{1118} flies. Progeny from these crosses were placed in vials containing 1% agar, and the number of dead flies were counted every 8 hrs. Flies were either heatshocked (HS) for one hour daily for induction of the HS-Gal4 or they remained at a constant temperature (NHS). Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The light blue, pink, blue and red arrows mark the mean lifespan of the double transgenic with heatshock, the single transgenic with heatshock, the double transgenic, and the single transgenic flies, respectively.
Figure 5.8 Males expressing RNAi directed towards the core domain do not have a change in starvation resistance compared to males not expressing core RNAi. Flies with the core RNAi construct were mated to either HS-Gal4 flies or w^{1118} flies. Progeny from these crosses were placed in vials containing 1% agar, and the number of dead flies were counted every 8 hr. Flies were either heatshocked (HS) for one hour daily for induction of the HS-Gal4 or they remained at a constant temperature (NHS). Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The light blue, pink, blue and red arrows mark the mean lifespan of the double transgenic with heatshock, the single transgenic with heatshock, the double transgenic, and the single transgenic flies, respectively.
Figure 5.9 Females expressing RNAi directed towards Z1 have a significant change in starvation resistance compared to females not expressing Z1 RNAi. Flies with the BRC-Z1 RNAi construct were mated to either HS-Gal4 flies or w^{1118} flies. Progeny from these crosses were placed in vials containing 1% agar, and the number of dead flies were counted every 8 hr. Flies were either heatshocked (HS) for one hour daily for induction of the HS-Gal4 or they remained at a constant temperature (NHS). Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The light blue, pink, blue and red arrows mark the mean lifespan of the double transgenic with heatshock, the single transgenic with heatshock, the double transgenic, and the single transgenic flies, respectively.
Figure 5.10 Males expressing RNAi directed towards Z1 did not have a significant change in starvation resistance compared to males not expressing Z1 RNAi. Flies with the Z1 RNAi construct were mated to either HS-Gal4 flies or w \textsuperscript{1118} flies. Progeny from these crosses were placed in vials containing 1% agar without food and the number of dead flies were counted every 8 hr. Flies were either heatshocked (HS) for one hour daily for induction of the HS-Gal4 or they remained at a constant temperature (NHS). Results shown are the mean ± S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The light blue, pink, blue and red arrows mark the mean lifespan of the double transgenic with heatshock, the single transgenic with heatshock, the double transgenic, and the single transgenic flies, respectively.
Double transgenic males have a mean lifespan of 75.3 +/- 4.8 and 82.2 +/- 3.6 hours when heatshocked daily or kept at constant temperatures, respectively. The mean starvation survival of RNAi BRC-Z1 single transgenic males is 80.9 +/- 0.6 and 85.7 +/- 1.5 hours when heatshocked daily or kept at constant temperatures, respectively.

5.3.3 Expression of RNAi BRC-Z2 Did Not Alter Starvation Resistance

Females transgenic for both HS-Gal4 driver and the RNAi BRC-Z2 transgene have a mean lifespan of 88.7 +/- 2.9 hours and 94.2 +/- 3.6 hours with and without daily heatshock, respectively (fig. 5.11). This is not a significant difference when compared to females that carry only the RNAi BRC-Z2 transgene which have a mean lifespan of 99.5 +/- 5.8 and 104.6 +/- 5.5 hours with and without daily heatshock respectively. The double transgenic males exposed to heatshock or kept at constant temperature had a decrease in mean starvation lifespan (71.8 +/- 3.4 and 71.5 +/- 1.3 hours, respectively) compared to the single transgenic RNAi BRC-Z2 males with and without daily heatshock, 79.5 +/- 1.4 and 81.8 +/- 0.2 hours, respectively (fig. 5.12). Since there is no change in starvation survival in the double transgenic when exposed to heatshock or kept at constant temperature, one could argue that the decrease in lifespan observed in the double transgenic is not caused by the expression of RNAi BRC-Z2. Decreased expression of BRC-Z2 by RNAi directed towards BRC-Z2 does not alter starvation survival compared to flies not expressing the BRC-Z2 transgene.
Figure 5.11 Females expressing RNAi directed towards BRC-Z2 do not have a significant change in starvation resistance compared to females not expressing Z2 RNAi. Flies with the Z2 RNAi construct were mated to either HS-Gal4 flies or $w^{1118}$ flies. Progeny from these crosses were placed in vials containing 1% agar, and the number of dead flies were counted every 8 hr. Flies were either heatshocked (HS) for one hour daily for induction of the HS-Gal4 or they remained at a constant temperature (NHS). Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The light blue, pink, blue and red arrows mark the mean lifespan of the double transgenic with heatshock, the single transgenic with heatshock, the double transgenic, and the single transgenic flies, respectively.
Figure 5.12 Males expressing RNAi directed towards BRC-Z2 do not have a significant change in starvation resistance compared to males not expressing Z2 RNAi. Flies with the Z2 RNAi construct were mated to either HS-Gal4 flies or w^{1118} flies. Progeny from these crosses were placed in vials containing 1% agar, and the number of dead flies were counted every 8 hr. Flies were either heatshocked (HS) for one hour daily for induction of the HS-Gal4 or they remained at a constant temperature (NHS). Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The light blue, pink, blue and red arrows mark the mean lifespan of the double transgenic with heatshock, the single transgenic with heatshock, the double transgenic, and the single transgenic flies, respectively.
5.3.4 Expression of RNAi BRC-Z4 Decrease Starvation Resistance in Females

A decrease in starvation lifespan in female flies that expressed RNAi BRC-Z4 was observed. Females transgenic for both the HS-Gal4 driver and the RNAi BRC-Z4 transgene exhibit a mean lifespan under starvation of 91.8 +/- 4.8 and 105.7 +/- 3.5 hours with and without daily heatshock, respectively (fig. 5.13). In contrast, females carrying only the RNAi BRC-Z4 transgene have a mean starvation lifespan of 106.2 +/- 3.4 and 112.8 +/- 3.2 hours, respectively. Males do not have altered starvation resistance when expressing BRC-Z4 RNAi. Males possessing both the HS-Gal4 transgene and the RNAi BRC-Z4 transgene have a mean lifespan under starvation of 87.3 +/- 2.4 hours and 94.7 +/- 1.3 hours with and without daily heatshock, respectively (fig. 5.14); while males with only the RNAi BRC-Z4 transgene have a mean starvation lifespan of 90.2 +/- 2.0 hours and 93.7 +/- 2.0 hours with and without heatshock respectively. As seen with RNAi BRC-Z1 expression, targeting BRC-Z4 expression decreases starvation survival in females only, when compared to those animals not expressing RNAi targeting BRC-Z4 expression.
Figure 5.13 Females expressing RNAi directed towards BRC-Z4 have a decrease in starvation resistance compared to females not expressing Z4 RNAi. Flies with the Z4 RNAi construct were mated to either HS-Gal4 flies or w^{1118} flies. Progeny from these crosses were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. Flies were either heatshocked (HS) for one hour daily for induction of the HS-Gal4 or they remained at a constant temperature (NHS). Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The light blue, pink, blue and red arrows mark the mean lifespan of the double transgenic with heatshock, the single transgenic with heatshock, the double transgenic, and the single transgenic flies, respectively.
Figure 5.14 Males expressing RNAi directed towards BRC-Z4 do not have a significant change in starvation survival compared to males not expressing Z4 RNAi. Flies with the Z4 RNAi construct were mated to either HS-Gal4 flies or w\textsuperscript{1118} flies. Progeny from these crosses were placed in vials containing 1% agar and the number of dead flies counted every 8 hrs. Flies were either heatshocked (HS) for one hour daily for induction of the HS-Gal4 or they remained at a constant temperature (NHS). Results shown are the mean +/- S.E.M. of data from three separate experiments, which for each contain 5 vials of 20 flies for each fly line. The light blue, pink, blue and red arrows mark the mean lifespan of the double transgenic with heatshock, the single transgenic with heatshock, the double transgenic, and the single transgenic flies, respectively.
5.4 Discussion

The decrease in BR-C expression by RNAi implies that BR-C expression is important for stress response. The pupal arrest phenotypes observed with all of the Act-5 Gal4 driven RNAi constructs suggest that the RNAi constructs reduce BR-C expression levels. The different pupal death phenotypes for different RNAi constructs support the idea that the RNAi constructs target only the specified BR-C isoform. This needs to be confirmed through molecular analysis of the RNA expression level of each isoform in transgenic lines for each RNAi construct. The expression of RNAi targeting the core sequence of BR-C should lower the expression levels of all BR-C isoforms. If the constructs are specific, RNAi BRC-Z1, BRC-Z2, BRC-Z3, and BRC-Z4 transgenics will show lower expression of the targeted isoform, but wild type expression of all other BR-C isoforms. This confirmation should be done on third instar larva since all BR-C isoforms are abundant at that time.

Because of the pupal arrest phenotype of the Act-5 Gal4 driven RNAi construct, a heatshock inducible Gal4 driver was used to express BR-C RNAi in adults to test starvation resistance. Based on the starvation data of the c754 mutant, it was expected that the expression of RNAi BRC-Z1, BRC-Z2, BRC-Z4, and core would cause an increase in starvation survival. The underexpression of BRC-Z1 and BRC-Z4 in females by RNAi decreased lifespan (table 5.1). The RNAi results for targeted lower expression of BRC-Z1 and BRC-Z4 females seems to correlate with the heatshock BRC-Z1 and BRC-Z4 overexpression data in Chapter 3 giving the opposite effect to starvation survival. The decrease in BR-C expression by RNAi resulting in a decrease in lifespan
<table>
<thead>
<tr>
<th>RNAi Construct</th>
<th>Double Transgenic</th>
<th>Single Transgenic</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heatshock</td>
<td>No Heatshock</td>
<td>Heatshock</td>
</tr>
<tr>
<td>Core</td>
<td>103.9 +/- 6.5</td>
<td>110.9 +/- 1.6</td>
<td>110.6 +/- 3.4</td>
</tr>
<tr>
<td>BRC-Z1</td>
<td>91.0 +/- 0.2</td>
<td>96.6 +/- 1.0</td>
<td>99.9 +/- 2.9</td>
</tr>
<tr>
<td>BRC-Z2</td>
<td>88.7 +/- 2.9</td>
<td>94.2 +/- 3.6</td>
<td>99.5 +/- 5.8</td>
</tr>
<tr>
<td>BRC-Z4</td>
<td>91.8 +/- 4.8</td>
<td>105.7 +/- 3.5</td>
<td>106.2 +/- 3.4</td>
</tr>
</tbody>
</table>

Table 5.1 Summary of lifespan under starvation of flies expressing BR-C RNAi compared to controls. The double transgenics are flies with the respective BR-C RNAi transgene and a Hs-Gal4 driver. The single transgenics are control flies that posses only a single BR-C RNAi transgene. Double and single transgenic flies were exposed to a one-hour daily heatshock for induction of Gal4.
disputes the argument that the increased starvation phenotype of c754 is caused by a decrease in *BR-C* expression. However, it is not known how the c754 mutation alters *BR-C* expression in various tissues. It may be that c754 has an increase in *BR-C* expression in the ovaries, but not in the whole body. One could also speculate that ubiquitous lowered expression of *BR-C* by RNAi alters other important functions besides those involved in stress resistance, therefore terminating life early. This could be tested by using tissue-specific Gal4 drivers.

A major problem with these experiments is that the time course of starvation experiments is very prolonged, making it difficult to measure the effect of Gal4/RNAi expression on mRNA levels. Unlike the Act-5 Gal4 driver, which is constitutively active, HS-Gal4 requires heatshock for expression of Gal4 causing Gal4 expression levels to decrease overtime after heatshock, which may result in lowered efficiency of silencing of the target RNA. It is plausible that in order for silencing to be effective the level of the RNAi expression must remain constant. Confirming the *BR-C* expression levels during RNAi expression at various time points after heatshock would address whether does Gal4 expression levels need to remain constant for effective RNAi silencing of the target gene.

If levels of RNAi need to remain constant, another dilemma arises. Most constitutively active drivers are expressed during larval development. As seen when *BR-C* RNAi is expressed using the Act-5-Gal4, larva do not survive to adulthood. To overcome this problem, three alternative inducible Gal4 systems can be employed: the pSwitch system, Tet-O system, and TARGET system (Han et al., 2000; McGuire et al., 2003; McGuire et al., 2004; Osterwalder et al., 1998; Roman and Davis, 2002; Stebbins et al., 2001; Stebbins and Yin, 2001). The first two systems, pSwitch and Tet-O, induce
Gal4 expression under RU486 or tetracycline food treatments, respectively. The TARGET system involves Gal80 inhibition of Gal4. At low temperatures, 18-19°C, Gal80 inhibits Gal4 transcription, moving the flies to 30°C would permit Gal4 activation of transcription. This will introduce another stress in the starvation assay that should be addressed when determining the response of the RNAi BR-C constructs. The TARGET system would be the best system for expressing the BR-C RNAi constructs because one can compensate for the increased heat while exposing the flies to another stress. In addition to being able to compensate for changes in temperatures when exposing flies to other stresses, there are many tissue specific Gal4 drivers available that could be used in this system for tissue specific RNAi BR-C expression.

In addition to fluctuating Gal4 levels with Hs-Gal4 transgenic flies, it is assumed that Gal4 is expressed globally. The Gal4 expression pattern can be determined by mating Hs-Gal4 transgenic flies to either UAS-GFP or UAS LacZ transgenic flies. Double transgenic progeny from this cross will express GFP or LacZ wherever Gal4 is expressed.
Chapter 6: Discussion and Future Work

6.1 Discussion

The hypothesis that I have tested during my thesis research is the expression of the ecdysone target gene, Broad-Complex (BR-C), is inversely proportional to the stress resistance and longevity in Drosophila melanogaster. The evidence presented in this thesis support the idea that changes in expression of BR-C can alter stress response and longevity. The data are complex: some experiments showing a positive correlation between BR-C expression and stress response and other experiments showing an inverse correlation to BR-C expression and stress response.

The data gathered thus far on the c754 mutant support the idea that BR-C mediates sensitivity to starvation and a shorter lifespan. The c754 mutant has increased lifespan and starvation tolerance, a phenotype that correlates with a decrease in BR-C expression: Yet the work done on c754 is incomplete. One needs to eliminate the possibility that the increased starvation survival and longevity seen in c754 mutants is the indirect result of the complex phenotype of altered BR-C expression such as reduced reproductive capabilities or delayed development. Furthermore, the Q-PCR analysis of expression of the BR-C isoforms in c754 is preliminary. The comparative C_T method used to analyze the Q-PCR data on BR-C expression in c754 compared to the control is not a valid method for analyzing the Q-PCR results because the efficiency to the BR-C reactions are not equal to the reactions of the housekeeping gene, RpL27. New primers or different starting template concentration may resolve this efficiency problem. One can also use the standard curve method to analyze the Q-PCR results to avoid the efficiency
problem. The Q-PCR reactions need to be further optimized plus additional Q-PCR assays determining \( BR-C \) expression in \( c754 \) compared to the control under varying conditions such as starvation and over time during lifespan needs to done. Tissue specific expression levels of \( BR-C \) also need to be determined in \( c754 \) and compared to the control line. The methods used to determine how \( BR-C \) expression is altered in \( c754 \) are inconclusive, but one can speculate why \( c754 \) alters \( BR-C \) expression.

The \( c754 \) P-element is located in an intron of the \( BR-C \) located between the P167 promoter and the ATG start site. In that same region of the \( BR-C \), Gonzy et al found two regulatory sites for the \( BR-C \) (Gonzy et al., 2002). One of these regulatory regions regulates \( br^+ \) (BRC-Z2) expression because a deletion spanning the regulatory site failed to complement the \( br^{28} \) allele, but did complement the other two \( BR-C \) complementing alleles. The other regulatory region regulates \( 2bc^+ \) (BRC-Z3) expression because a deletion spanning the regulatory region failed to complement the \( 2bc' \) allele, but complemented the other \( BR-C \) allele groups. Interestingly the deletion in the genome in which the \( 2bc^+ \) regulatory region is located spans the presumed regulatory site of \( br^+ \): Yet this deletion complements the \( br^{28} \) allele. The information provided indicates that there may be regulatory regions for both \( br^+ \) and \( 2bc^+ \) in the 5' the intronic region of the \( BR-C \) where \( c754 \) is located. One can speculate that the \( c754 \) insertion alter \( BR-C \) expression by disrupting one or many of these regulatory sites.

Data on the \( c754 \) mutant concerning \( BR-C \) mediating starvation tolerance and lifespan conflicts with the increased survival under starvation conditions observed with \( BR-C \) overexpression. It is intriguing that both \( BR-C \) underexpression and overexpression lead to increased starvation resistance. One possible explanation is that
c754 expression data is inaccurate and thus the hypothesis is incorrect and should state that increased BR-C expression causes increased lifespan and stress tolerance. Alternatively, the BR-C isoform that leads to stress resistance and increased lifespan in c754 may be globally repressed, but increased in expression in tissues of the fly important for stress response. Another explanation is overexpression of one BR-C isoform may inhibit or reduce the expression of another BR-C isoform. One can infer that the increase in starvation survival seen with overexpression of one BR-C isoform may cause a decrease in overall BR-C expression levels. Confirming BR-C expression levels in the BR-C overexpressing lines and determining the tissue specific expression of BR-C in c754 is necessary to determine how BR-C expression mediates stress response.

The result from the experiments in which RNAi decreases BR-C expression levels supports the hypothesis that altered expression of BR-C causes a change in starvation resistance. When flies expressing RNAi toward one BR-C isoform are exposed to starvation conditions, the flies have a decrease in survival. In conjunction with the overexpression data, this result suggests a direct correlation between BR-C expression and stress response. A new hypothesis would be needed to explain the c754 mutant behavior if a direct correlation exists. Although the RNAi data implies that decreased BR-C expression causes a decrease in starvation survival, these data are not conclusive for several reasons: ubiquitous overexpression of RNAi, insufficient controls, and lack of molecular analysis. Ubiquitous overexpression of BR-C RNAi may not be sensitive enough because different levels of BR-C expression may be needed in different tissues to contribute to the stress response. Tissue specific expression of BR-C RNAi using the TARGET system would address this issue. The next issue, insufficient controls, is
addressed by analyzing the starvation phenotype of flies with the HS-Gal4 driver as the only transgene. This control is important because there may be genetic contribution from the HS-Gal4 parent to the starvation phenotype. Furthermore, molecular analysis of BR-C expression level in BR-C RNAi transgenic lines needs to be performed. The pupal arrest phenotype observed in the Act-5 Gal4 expression of BR-C RNAi implies that the BR-C RNAi decreases BR-C expression, but the extent and spatiotemporal characteristics of the decrease are unknown. Furthermore, an inducible constitutively active Gal4 driver such as the TARGET system needs to be employed for BR-C RNAi expression in adults. Until the expression levels of BR-C is determined in the BR-C RNAi lines, the data gathered in regards to starvation survival is not interpretable.

Thus far, the work presented supports the hypothesis that BR-C expression mediates stress resistance and longevity. The role of BR-C in mediating stress response remains undetermined.

It could be argued that the effect of BR-C is an indirect effect. The BR-C has an established role in metamorphosis; Altered BR-C expression during metamorphosis may cause developmental defects, which could cause altered stress resistance and longevity. A developmental defect caused by BR-C that alters stress resistance and longevity would be considered an indirect effect of altered BR-C expression. It was observed that the BR-C misexpressing mutant, c754, and the heatshock overexpressing BR-C transgenic lines have delayed development. The delayed development is the result of longer eclosion-times compared to their controls. The delayed development may cause the third instar larval stage to last longer therefore allowing for the consumption of more food. This increased consumption of food can increase nutrient storage, allowing the altered
BR-C expressing lines to live longer under starvation conditions. I would argue that
BR-C has a direct effect because a change in starvation resistance occurs with induced
expression of BR-C RNAi, which occurs after development.

To test the direct of BR-C on stress response and longevity, transcript and protein
levels of BR-C should measured in wild type flies under normal conditions and stressful
conditions. This would not only emphasize the direct role of BR-C in stress resistance
and longevity, but it would also be useful in determining whether the effect of BR-C is
positively or inversely correlated to stress resistance and longevity.

The direct effect of BR-C on stress response strengthens the model of steroid
mediated stress response and longevity. Based on prior work in C. elegans D.
melanogaster, and mice, a conserved model for aging can be developed. Both steroid
and insulin signaling influence aging. I will develop a model for steroid signaling. Upon
sensing stressful conditions, the level of the steroid hormone involved decreases. In C.
elegans, there are no known steroid hormones. The Drosophila steroid hormone is
ecdysone. Mammals have several steroid hormones, based on its role in reproduction; I
would surmise that estrogen would be the steroid hormone involved in stress response
and longevity. The decreased level of steroid hormone causes the nuclear hormone
receptor, such as DAF-12 in C. elegans and EcR in Drosophila, to become inactive.
When inactivate, the steroid hormone receptor cannot induce the transcription of a
BTB/POZ C2H2 zinc finger transcription factor. BTB/POZ C2H2 zinc finger transcription
factors are common throughout the animal kingdom; my data suggest that BR-C is this
transcription. Decreased expression of the BTB/POZ C2H2 zinc finger transcription
factor induces a forkhead transcription; BR-C inhibits the HNF-3/fkh fork head
transcription factor (Renault et al., 2001). In C. elegans, DAF-16 is a fork-head transcription factor that becomes active during stress. Once activated the fork-head transcription factor induces genes that cause stress resistance and longevity.

6.2 Future Work

To test the concept that BR-C mediates stress response, further work needs to be done. First, additional stress tests such as heat and oxidative stress tolerance need to be done with c754 mutants, BR-C overexpressing lines, and RNAi mediated BR-C underexpressing lines. This is important because it determines the extent to which BR-C participates in stress response. In addition to more stress tests done on c754, the molecular techniques, i.e. Q-PCR, needs to be further optimized to determine how c754 is altering BR-C expression. Molecular work also needs to be done on the heatshock overexpression and RNAi mediated underexpression lines of the BR-C. Molecular analysis of these lines will give insight to how altered expression of one BR-C isoforms influences expression of the other BR-C isoforms. This will aid in determining if expression of BR-C is directly related to or inversely related to stress response.

It is necessary to determine if the starvation phenotype of the BR-C expression lines is really due to BR-C misexpression and not the result of diminished reproductive capabilities. This is important to test because if reproductive capabilities are decreased because of altered BR-C expression, the fly may have more stored resources available to withstand starvation conditions longer: Therefore, the increased starvation resistant phenotype is an artifact of reduced reproduction caused by BR-C and not from BR-C mediating stress response. Preliminary observations of c754 cultures and BR-C
heatshock overexpression cultures show that these lines have a decrease in the number of progeny compared to control lines suggesting a possible defect in reproduction. To test this, one needs to examine the amount of eggs that are laid and the number of progeny that emerge. If decreased fertility is determined for all of the altered BR-C expression lines, then one needs to compare the starvation phenotypes of these altered BR-C expressing lines to flies with reduced reproductive capabilities such as the female sterile, ovo\textsuperscript{D1}. More alleles of the BR-C need to be tested for stress survival to further solidify the role of BR-C in stress response and lifespan and to determine if the relationship between BR-C expression, stress response and lifespan is, direct or opposite.

Most alleles of the BR-C arrest development before or during pupation, making it difficult to study stress response in adults, but there are some mutants that survive to adulthood. These mutants are \textit{br\textsuperscript{l}}, \textit{br\textsuperscript{3}}, \textit{br\textsuperscript{27}}, and \textit{br\textsuperscript{rbp-2}}. All of these mutations are homozygous viable and hypomorphic (Belyaeva et al., 1980; Belyaeva et al., 1982; Emery et al., 1994; Gotwals and Fristrom, 1991; Restifo and Merrill, 1994; Sampedro et al., 1989). Hypomorph mutations have reduced activity of the mutant gene. \textit{br\textsuperscript{l}} and \textit{br\textsuperscript{3}} have shorter and wider wings: some \textit{br\textsuperscript{3}} flies have crumpled arc like wings (Belyaeva et al., 1980; Belyaeva et al., 1982). \textit{br\textsuperscript{27}} flies have no visual phenotype but there is a reduction in dorso-ventral indirect flight muscles (Restifo and Merrill, 1994). \textit{br\textsuperscript{rbp-2}} flies have fewer than normal bristles on the palpus (Gotwals and Fristrom, 1991). The \textit{br\textsuperscript{l}}, \textit{br\textsuperscript{3}}, \textit{br\textsuperscript{27}}, and \textit{br\textsuperscript{rbp-2}} alleles are all deficient in \textit{rbp\textsuperscript{+}} (BRC-Z1 isoform) function (Kiss et al., 1988). Furthermore, the \textit{br\textsuperscript{l}} allele is viable when heterozygous with some of the BR-C null mutants. Testing the survival of these mutants during stress conditions would further support the role of the BR-C as a mediator of stress response and may aid in
determining which isoforms play a key role in the process. If a stress phenotype is
determined for hypomorphic mutants of BR-C, then expression levels of all the BR-C
isoforms should be determined for each of the hypomorphic mutants. Kiss et al. found
that these mutants have reduced rbp⁺ expression in adults based on phenotype (Kiss et al.,
1988). However, molecular analyses on these BR-C mutants were not done. If these
mutants of BR-C have an increase in starvation survival and decreased BR-C expression,
then this finding would support the hypothesis of decreased BR-C expression increases
stress survival and lifespan.

In addition to testing, more BR-C mutants for their stress response phenotype, the
induction or repression of the BR-C isoforms in wild type flies when exposed to stress
should be determined. If the BR-C mediates stress response, then there should be some
altered expression of the BR-C observed in wild type flies when exposed to stressful
conditions. RNA samples from wild type flies at various starvation exposure times
should be collected and expression levels of BR-C should be determined.

Once the role of BR-C, as a mediator of stress response has been established then
one needs to test the effect of ecdysone in altering BR-C expression in adult flies. One
can feed ecdysone to wild type flies (Simon et al., 2003) and determine stress phenotypes
and BR-C expression profiles after consumption of ecdysone. If the hypothesis is correct,
then flies fed ecdysone should cause a decrease in stress response and an increase in BR-
C expression. Epistatic stress experiments with the ecdysone mutants and BR-C mutants
could be done to determine if the increase in stress resistance seen in the ecdysone
receptor mutants and the ecdysone synthesis mutants is mediated by BR-C expression. If
the increased stress resistance seen in the ecdysone receptor mutants and the ecdysone
synthesis mutant is caused by reduced expression of BR-C, then there should be no change in the stress survival of the double mutants, which have mutations in ecdysone signaling and altered BR-C expression compared to the single ecdysone signaling mutants and BR-C mutants.

In addition to testing the upstream ecdysone-signaling pathway, the expression of the downstream targets of the BR-C needs to be examined during stress response. BR-C induces the expression of the small heat shock protein 23. Flies bred for longevity have increased expression of shsp23 and increased stress resistance (Kurapati et al., 2000). It may be that the BR-C mediates stress response via regulation of shsp23. The small heatshock protein expression level would be an interesting place to look as a possible downstream target of BR-C mediated stress response.

After confirming BR-C as the downstream target of the ecdysone regulated stress response pathway, it would be interesting to examine if tissue specific expression of the BR-C isoforms makes a difference. In C. elegans, the gonad has a role in regulating stress response. Using the Gal4/UAS expression system, overexpression of the BR-C isoforms and underexpression of BR-C isoforms in the ovaries of the fly may provide some interesting results and validate that the gonad in Drosophila melanogaster mediates stress response.
References:


