DETERMINING THE CORRELATION BETWEEN STRUCTURE AND FUNCTION FOR THE DROSOPIHLA INSULIN RECEPTOR SUBSTRATE \textit{chico}

by

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Abstract

The *Drosophila* gene *chico* codes for the homolog of the vertebrate insulin receptor substrate (IRS) family of proteins. CHICO is phosphorylated by an activated insulin receptor (IR) and signals to several downstream signalling pathways through various predicted binding domains.

To investigate how the structure of CHICO contributes to its function, an ethyl methanesulfonate (EMS) screen was performed to generate novel *chico* alleles. One novel allele, *chico*\(^{13063}\), was recovered out of 22,072 lines screened. This allele displayed numerous *chico* phenotypes including: reduced body size, weight, wing size, and developmental time delay. These phenotypes were stronger in the *chico*\(^{13063}\) allele than the null allele, *chico*\(^{1}\) and the deficiency, *Df(2L)flp 147E*. The *chico*\(^{13063}\) coding region was sequenced and no genetic lesions were found, suggesting the mutation is in the regulatory region. Future work would be to sequence putative regulatory regions of *chico* and examine the expression levels of CHICO in the *chico*\(^{13063}\) mutant.

Key Words: *Drosophila*; insulin signalling; insulin receptor; insulin receptor substrate; *chico*
Dedication

For my sister, without whom I would never have been able to do this.
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1: Introduction

1.1 Insulin Signalling Pathway

On a cellular level, organisms develop and maintain homeostasis by numerous complex signalling pathways. The insulin/insulin-like growth factor (IGF) signalling pathway (IIS) is one such pathway and is known to have diverse functions in development (Kimura et al., 1997), growth and size (Butler and Le Roith, 2001), aging (Clancy et al., 2001; Kenyon, 2005), metabolic homeostasis (Saltiel and Kahn, 2001), stress resistance (Murakami and Johnson, 1996), reproduction (Arik et al., 2009) and immunity (Evans et al., 2008). Because of this diversity, alterations in the IIS can have severe detrimental effects such as diabetes mellitus (Nandi et al., 2004) and certain forms of cancer (Simpson and Parsons, 2001; Hafen, 2004).

The ability of the IIS pathway to be involved in such essential functions is mainly through extensive crosstalk with other signal transduction cascades such as the protein kinase TOR (target of rapamycin) pathway (Hietakangas and Cohen, 2009; Stanfel et al., 2009). The TOR pathway is the main mediator of cellular nutrient sensing and regulates protein synthesis and growth in response to amino acids and growth factors (Wullschleger et al., 2006). The kinase TOR complex 1 (TOR-C1) is the major rapamycin sensitive form of Tor and it is the central mediator of TOR pathway for nutrient sensing and growth control and interacts with numerous downstream proteins (Hietakangas and Cohen, 2009). An example of a core interaction between the TOR pathway and the IIS pathway is through phosphoinositide dependent kinase (PDK),
which can be phosphorylated by phosphotidylinositol (3,4,5) triphosphate (PIP\textsubscript{3}) from the IIS pathway and S6 kinase (S6K) from the TOR pathway (Giannakou and Partridge, 2007).

The core components of the IIS pathway include insulin/insulin-like peptides, the insulin receptor and substrate(s), the lipid kinase phosphoinositide 3-kinase (PI3K), the lipid phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10), the serine-threonine kinase PKB (protein kinase B) and forkhead transcription factors (Hafen, 2004) (Figure 1). Many of the components of the IIS pathway are highly conserved in metazoans (Piper et al., 2007) (Figure 2). Research has shown that single gene mutations in particular genes in the IIS pathway can extend lifespan in the mouse *Mus musculus* (Selman et al., 2008), the nematode worm *Caenorhabditis elegans* (Klass, 1983; Freidman and Johnson, 1988), and the fruit fly *Drosophila melanogaster* (Clancy et al., 2001), implying an evolutionary conservation of mechanisms (Chistyakova, 2008).
Figure 1: The Core Insulin Signalling Pathway for Mammals, *Drosophila* and *C. elegans*

The core proteins of the IIS pathway are shaded in grey with arrows and lines indicating interactions. The homologs of the worm, fly and mouse are indicated in the boxes attached to the protein by dashed lines. Diagram adapted from Broughton and Partridge, 2009.
Figure 2: Conservation of the Core IIS Pathway in Mammals, *Drosophila* and *C. elegans*

The conservation of the core proteins in the IIS pathway is shown at the various levels of the signalling cascade from the extracellular ligands to the forkhead transcription factors in the nucleus. Positive interactions are indicated by arrows and negative interactions by lines. Diagram adapted from Garofalo, 2002.
1.1.1 The Insulin/Insulin-like Peptides

In vertebrates, there are two types of ligands that activate the IIS pathway. The first type is insulin that is produced by the pancreatic β-cells. Insulin is secreted into the circulatory system in response to an increase in blood glucose levels where it binds to and activates the insulin receptor in various tissues (Myers and White, 1996). The immediate translation product of the insulin gene is a single polypeptide called preproinsulin containing a signal peptide at the N-terminus that targets it to the endoplasmic reticulum, where it is cleaved into three units (Chan et al., 1976). The resulting protein, proinsulin, is composed of two peptide chains (A and B) that are joined by a connecting C-peptide (Bell et al., 1980). Subsequent processing of proinsulin results in the cleavage of the C-chain and disulphide linkage of the A and B chains to form mature insulin (Bell et al., 1980). The second group of ligands is the insulin-like growth factors 1 and 2 that are mainly produced by the liver with only a minor contribution from the brain and which are involved in regulating organismal growth in response to growth hormones (de la Monte and Wands, 2005). IGF-1 and IGF-2 share approximately 50% amino acid identity with insulin with the main structural difference being the retention of the C chain that is cleaved from proinsulin (de la Monte and Wands, 2005). Unlike insulin, IGFs in circulation are bound by high-affinity binding proteins (IGFBP) that function in stabilizing and prolonging the half-life of IGFs (Jones and Clemmons, 1995). Insulin and IGF-1/2 can bind to and activate the insulin receptor (IR), the IGF receptor (IGF-1R), or a hybrid of the IR and IGF-1R (Taguchi and White, 2008).
Drosophila has seven insulin-like peptides termed dilp1-7 for Drosophila insulin-like peptides. The ligands are structurally similar to mammalian preproinsulin, with a signal peptide, a C peptide, and A and B chains (Brogiolo et al., 2001; Seidah and Chretien, 1997), as well as being functionally equivalent. Mammalian insulin is able to activate the Drosophila INR (Yamaguchi et al., 1995) and Drosophila protein extract shows signalling activity in the mouse (Meneses et al., 1975). Each DILP has a different expression pattern and unique regulation, suggesting that the functions of the various DILPs are not overlapping (Teleman, 2010). Four of the DILPs: DILPs 1, 2, 3 and 5, are expressed in seven median neurosecretory cells (m-NSCs) in the brain that possess axon terminals in the larval endocrine gland and on the aorta where DILPs may be released into the circulatory system (Cao and Brown, 2001; Ikeya et al., 2002; Broughton et al., 2005). DILPs 4, 5 and 6 are expressed in the midgut, DILP 2 in the imaginal discs and the salivary gland, and DILP 7 in the ventral nerve cord of the brain (Brogiolo et al., 2001; Ikeya et al., 2002). Overexpression of any of the seven DILPs during development leads to an increase in body size, which would indicate that all seven ligands can activate the insulin receptor; however, distinct functions for each DILP have yet to be elucidated (Teleman, 2010).

In C. elegans, there are believed to be as many as 38 insulin-like genes that express insulin-like peptides (INS) in many tissues throughout the body. Several have been shown to act as receptor agonists or antagonists (Pierce et al., 2001), however, the functions of many are widely unknown. Insulin-like gene 1 (INS-1) is the most closely related to human insulin in terms of primary sequence similarity and structural homology and is one of two INS proteins that contain a cleavable C chain peptide (Panowski and
INS-1 seems to function as an antagonist of the insulin receptor DAF-2 (abnormal *dauer* formation-2) in that overexpression of INS-1 results in decreased DAF-2 signalling (Pierce *et al.*, 2001). DAF-28 is an insulin-like peptide that functions as an agonist in that worms expressing a dominant negative mutant allele of DAF-28 display many traits indicative of decreased DAF-2 signalling (Li *et al.*, 2003).

### 1.1.2 The Insulin Receptor

Many of the signalling components in mammals consist of multiple isoforms (Benyoucef *et al.*, 2007) and have tissue specific expression patterns, which builds the potential for many layers of IIS complexity (Thirone *et al.*, 2006). For example, the insulin/IGF-1/2 ligands bind to and activate five different isoforms of the insulin receptor (IR): two insulin receptor isoforms, IRα and IRβ; the insulin-like growth factor receptor, IGF1R; and two hybrid receptors, IRα::IGF1R and IRβ::IGF1R (White and Kahn, 1994). The IR is derived from a single proreceptor that is cleaved into an α-subunit and a β-subunit that are linked to each other by disulfide bonds and assemble as heterotetramers with another α/β subunit (Taguchi and White, 2008). The different isoforms of the IR are due to one site of alternative splicing within the sequences encoding the α-subunit that results in two receptor isoforms differing by 12 amino acids (White and Kahn, 1994). The α-subunits are located extracellularly and contain cysteine rich insulin binding sites. The β-subunits contain a transmembrane domain and several intercellular tyrosine kinase domains (Taguchi and White, 2008). The IGF-1R is structurally similar to the IR in that it shares 84% amino acid sequence identity in the β-subunits; however, homology is lower in the extracellular domains of the α-subunits (48%), which likely defines ligand specificity for IGF-1 (Ullrich *et al.*, 1986). Both receptors undergo multi-site tyrosine
auto-phosphorylation of the receptor and subsequently recruit the insulin receptor substrates (IRS1-4).

The *Drosophila* insulin receptor (INR) is somewhat different compared to the mammalian insulin and IGF1 receptors. The *Drosophila* INR proreceptor consists of an insulin-binding α subunit and a β subunit with a protein tyrosine kinase domain (Fernandez *et al*., 1995) and undergoes proteolytic cleavage to produce an α subunit containing the ligand binding domains and a transmembrane β subunit, which then form heterotetramers (Brogiolo *et al*., 2001). The INR also contains a 400-amino acid (60kDa) C-terminal extension attached to the β subunit not found in any of the vertebrate receptors that produces a novel domain consisting of multiple tyrosine autophosphorylation sites (Fernandez *et al*., 1995). These sites consist of three YXXM consensus binding sites for the SH2 domain of the p60 subunit of PI3K (Kappeller and Cantley, 1994) and four additional NPXY consensus phosphotyrosine (PTB)binding sites (Böhni *et al*., 1999). It is believed that the INR functions in two different ways upon ligand binding: the receptor autophosphorylates (Ruan *et al*., 1995) and either recruits the insulin receptor substrates (IRS) CHICO and LNK (Böhni *et al*., 1999; Werz *et al*., 2009) or signals through the docking sites in the C-terminal tail (Fernandez *et al*., 1995). Double mutations in *chico* and *lnk* are lethal, suggesting that these two proteins transduce the majority of the signal downstream of the INR (Teleman, 2010). The ability of the *Drosophila* INR to signal without the IRSs may be due to the differences in the C-terminal domain and may explain the phenotypic difference between mutations in the mouse IR versus *Drosophila* INR. Mutations in the mouse IR results in death shortly after birth, whereas heteroallelic combinations of mutations in the *Drosophila* INR produce viable offspring with a small
body phenotype and increased total body free sugar and lipid levels (Chen et al., 1996; Shingleton et al., 2005).

In *C. elegans* the insulin-like ligands bind to the insulin receptor DAF-2 and generate an intracellular signalling cascade. *daf-2* codes for the only insulin/IGF-1 receptor homolog in the worm (Kenyon et al., 1993). DAF-2 is structurally similar to the mammalian IR with two extracellular protein chains containing putative cysteine rich ligand binding domains and two intracellular protein chains containing transmembrane domains and several tyrosine kinase domains (Kimura et al., 1997). Like the *Drosophila* INR, DAF-2 has a long C-terminus extension containing a number of tyrosine autophosphorylation motifs including a PTB binding site and three SH2 binding sites for proteins such as the PI3K (Kimura et al., 1997). Mutations to *daf-2* result in larvae entering a dauer constitutive phase that increases life-span (Tissenbaum and Guarente, 2001).

1.1.3 Insulin Receptor Substrate

Signalling through the insulin receptor proceeds through substrate proteins known as insulin receptor substrates that function as docking proteins for Src-Homology 2 (SH2) domain containing proteins such as phosphoinositol 3-kinase (PI3K) (Lavan et al., 1997; Hafen 2004). Mammals have four insulin receptor substrates, IRS1-4 (Lavan, et al., 1997). All four IRS family members possess a common domain structure that includes a pleckstrin homology (PH) domain and a PTB domain at the N-terminus and a number of tyrosine phosphorylation sites in the C-terminus (Lavan et al., 1997). Several of the phosphotyrosine motifs in the C-terminus function as the binding sites for SH2 containing proteins. The insulin receptor substrate binds to the phosphorylated NPXY
motif of the insulin receptor or IGF-1R through the PTB domain (White, 2003). Work with IRS1-4 in mice has suggested that most insulin responses are mediated by IRS1 or IRS2, whereas the others play compensatory roles (Schubert et al., 2003).

*Drosophila* have two insulin receptor substrates, *chico* and *lnk*. The insulin receptor substrate *lnk* (*chico* will be discussed in detail in the following section) was predicted to exist by Kulansky Poltilove et al. in 2000 during their analysis of the insulin receptor substrate *chico*. They believed that because the *chico* phenotype was milder than the phenotype produced by mutations caused by a lack of the insulin receptor that there could be more than one protein that functions directly downstream of the insulin receptor. The presence of a second IRS was confirmed recently by the discovery of the SH2B family adaptor protein *lnk* (Werz et al., 2009). The *Drosophila* genome contains a single homolog for the three members of the mammalian SH2B family of proteins (Huang et al., 1995; Riedel et al., 1997; Yokouchi et al., 1997). *lnk* codes for a 723 amino acid adaptor protein containing a PH domain, an SH2 binding motif and a highly conserved C-terminal Cbl (*casitas B-lineage lymphoma*) recognition motif (Werz et al., 2009). Homozygous *lnk* mutants show a similar phenotype to the mutations in the other IRS *chico* in that they have a small body for all developmental stages due to reductions in cell number and cell size, females are sterile, and they have elevated levels of lipids (Werz et al., 2009). Epistasis experiments placed *lnk* at the same level as *chico*, functioning after the INR and before PI3K; additionally, double mutants were lethal implying that the two substrate proteins act independently downstream of the INR (Werz et al., 2009). *lnk* lacks the YXXM consensus binding site that *chico* contains (that binds to the SH2 domain of the regulatory subunit of PI3K) and therefore does not interact with
the downstream protein PI3K in the same way. The specific functional interactions of lnk are still unclear and it has been proposed that there are likely still more binding proteins that function in this intermediate step of the IIS pathway (Werz et al., 2009).

In mammals and Drosophila, after the binding of an insulin-like ligand to the receptor, an insulin receptor substrate binds to the receptor and propagates the signal in the cell. In C. elegans, there is a very divergent insulin receptor substrate called ist-1 (insulin receptor substrate-1) (Wolkow et al., 2002). The IST-1 protein structure does share some similarities to other insulin receptor substrates in that it has a PH and a PTB domain and may contain a potential docking site for SH2 domain-containing proteins. Experiments show that IST-1 can potentiate the IIS signal although it is not required for signalling under most conditions (Wolkow et al., 2002).

1.1.4 Drosophila Insulin Receptor Substrate chico

A screen for mutations causing a reduction in body size originally identified a P-element-induced mutation, fs(2)4’ (Berg and Spradling, 1991). Böhni et al. renamed this mutation chico, which means “small boy” in Spanish. The chico gene is located on chromosome 2, left arm, region 31B1, and is flanked by the genes basket (bsk), that encodes DJNK (Drosophila c-Jun N-terminal kinase) (Sluss et al., 1996) and maternal expression at 31B (me31B), that encodes a “DEAD box” (Asp-Glu-Ala-Asp) RNA helicase (de Valoir et al., 1991). The gene locus consists of a single transcription unit totalling 3.6 kb with nine exons with a putative translational start site in the second exon (Böhni et al., 1999) (Figure 3). The original chico mutant allele, chico1, contains a P-element insertion 80 base pairs downstream of the translation start site and is a proposed
null based on the location of insertion but it has not been molecularly characterized (Böhni et al., 1999) (Figure 3).

The open reading frame of CHICO is predicted to code for 968 amino acid residues and has a calculated molecular mass of 108 kDa (Kulansky Poltilove et al., 2000). The CHICO amino acid sequence displays a strong similarity with the vertebrate insulin receptor substrate proteins, IRS1-4 (Böhni et al., 1999). Sequence similarity between CHICO and IRS1-4 is confined to the N-terminal region that includes a PH domain and a PTB domain that are 45 and 41% identical to IRS-1, respectively (Böhni et al., 1999; Kulansky Poltilove et al., 2000) (Figure 4). CHICO interacts with the IR through the binding of its PTB domain with the juxtamembrane NPXY motif of the insulin receptor (Kulansky Poltilove et al., 2000). The C-terminal phosphorylation domain does not contain a significant overall homology with IRS1-4; however, the CHICO protein contains at least six potential sites of tyrosine phosphorylation that are present in consensus binding sequences for SH2 domain-containing proteins (Kulansky Poltilove et al., 2000) and is characteristic for IRS family members (Böhni et al., 1999). Two of the tyrosine phosphorylation sets are YXXM motifs that are present at positions

Figure 3: The chico Locus

chico is located on chromosome 2L and has nine exons. The open boxes indicate untranslated exons and the black boxes represent translated exons. The site of the P-element (fs(2)4') insertion in exon 2 is indicated. Adapted from Böhni et al., 1999.
411 and 641 and potentially form a site for interaction with the p60 adaptor subunit of p110 PI3K (Songyang et al., 1993; Weinkove et al., 1997).

Flies homozygous for the original chico\textsuperscript{1} allele are semi-lethal and flies that survive eclose 2-3 days after their heterozygous siblings and are female sterile (Böhni et al., 1999). As indicated by the name chico, a characteristic phenotype for the chico mutant is a smaller than average sized fly. The size reduction phenotype for homozygous chico\textsuperscript{1} was observed at all developmental stages from embryo to adult fly. Böhni et al. quantified the size reduction of the homozygous mutants using weight: female weight was reduced by 65% and males by 55% when compared with wild-type control flies of the same age. There was no reduction in weight for the heterozygous chico\textsuperscript{1}/+ fly. After performing cell size assays, it was concluded that the reduction in body size was due to a reduction in both cell number and cell size; of the total reduction, 68% was due to a reduction in cell number and 32% was due to a reduction in cell size and that CHICO functions in a cell autonomous manner (Böhni et al., 1999).
CHICO is also involved in cellular metabolism with \textit{chico} \textsuperscript{1} mutants having an almost 2-fold increase in lipid levels when compared to their heterozygous siblings (Böhni \textit{et al}., 1999). This phenotype is also observed in mice that are deficient for IRS-1 (Abe \textit{et al}., 1998) and \textit{C. elegans} having a mutation in \textit{daf-2}, an insulin receptor homolog (Kimua \textit{et al}., 1997). Based on these results it would seem that the IIS pathway is involved in controlling cellular metabolism in a variety of organisms from vertebrates to insects.

\textbf{1.1.5 Target Effectors of the Insulin Receptor Substrate}

After stimulation of the IIS pathway that results in the autophosphorylation of the INR and activation of the IRSs the next protein to be phosphorylated is phosphoinositol 3-kinase (PI3K/p110) and its adapter subunit p85 (p60 in \textit{Drosophila}) (Leevers \textit{et al}., 1996; Weinkove \textit{et al}., 1997). p110 is the catalytic subunit and p85/p60 is the regulatory subunit of PI3K (Giannakou and Partridge, 2007). Upon activation, the complex is recruited to the cell membrane where it then converts phosphatidylinositol (4,5)-bisphosphate (PIP\textsubscript{2}) to phosphatidylinositol (3,4,5)-trisphosphate (PIP\textsubscript{3}) by phosphorylating the inositol ring at the 3-position (Leevers \textit{et al}., 1996). The action of PI3K is antagonized by the phosphatase activity of the tumour suppressor PTEN (phosphatase and tensin homolog on chromosome 10), which degrades PIP\textsubscript{3} to PIP\textsubscript{2} (Goberdhan \textit{et al}., 1999). Accumulation of PIP\textsubscript{3} in the plasma membrane recruits two kinases, PDK (phosphoinositide-dependent kinase) and AKT2 (protein kinase B (PKB) in \textit{Drosophila}) to the cell surface (Giannakou and Partridge, 2007; Garofalo \textit{et al}., 2003). PDK contains two functional domains: a serine/threonine kinase domain and a PH domain that interacts with PIP\textsubscript{3} (Rintelen \textit{et al}., 2001). AKT2/PKB also contains a PH
domain that interacts directly with PIP$_3$ but it can also be activated through phosphorylation by PDK (Saltiel and Kahn, 2001). Phosphorylation of AKT2/PKB relieves intermolecular inhibition and allows for the dissociation of the activated AKT2/PKB from the membrane where it subsequently moves to the nucleus (Coffer et al., 1998). This elevated PIP$_3$, as a result of IIS signalling, can lead to increases in nuclear AKT2/PKB where it can affect transcriptional output.

Propagation of the insulin signal after activation of the receptor in C. elegans is similar to mammals and Drosophila. The DAF-2 receptor mainly signals through the direct activation of PI3K that consists of a p55-like subunit, AAP-1 (Wolkow et al., 2002) and a p110 catalytic subunit, AGE-1 (Morris et al., 1996). Mutations in daf-2 and age-1 lead to significant extensions of life span (Friedman and Johnson, 1988; Guarente and Kenyon, 2000; Panowski and Dillin, 2009).

The AAP-1/AGE-1 complex then phosphorylates PIP$_2$ into PIP$_3$ and is antagonized by 3-phosphatase activity of DAF-18, the worm homolog to PTEN (Ogg and Ruvkun, 1998). The PIP$_3$ signal activates PDK-1, the homolog of mammalian AKT2 (Paradis et al., 1998), which then phosphorylates AKT-1, AKT-2 and SGK-1 (Hertweck et al., 2004). SGK-1 (serum and glucocorticoid inducible kinase-1) is involved with development, stress response and longevity (Hamilton et al., 2005) while AKT-1 and AKT-2 are redundant and are primarily involved in dauer formation (Paradis and Ruvkun, 1998).
1.1.6 Forkhead Transcription Factors

In mammals the protein kinase AKT2 phosphorylates many downstream proteins including a family of three forkhead transcription factors: FKHR (forkhead in rhabdomyosarcoma/ also known as FOXO1), FKHR1 (FKHR-like 1) and AFX (ALL1 fused gene from chromosome 1) (Woods and Rena, 2002). Phosphorylation of the forkhead transcription factors results in their inactivation and sequestration in the cytosol, which alters the expression of many genes involved in apoptosis, the cell cycle, DNA damage repair, oxidative stress, cell differentiation, glucose metabolism and other cellular functions (Huang and Tindall, 2007).

*Drosophila* has a single forkhead transcription factor homolog FOXO (forkhead box O) that contains three PKB phosphorylation consensus sites and a conserved DNA binding domain (Neufeld, 2003). Phosphorylation of FOXO by PKB in response to insulin results in the inhibition of FOXO and its export from the nucleus to the cytoplasm and subsequent transcriptional inactivation (Puig *et al*., 2003). FOXO is predicted to regulate at least 2000 genes (Gershman *et al*., 2007). Among these are many components of the translational apparatus, as well as, mitochondrial components (Teleman, 2010). One target of FOXO is the translational repressor 4E-BP (4E-binding protein) that binds eIF4E (eukaryotic initiation factor 4E) and blocks recruitment of the ribosome to the 5’ end of mRNAs that results in a decrease in cellular translation rates and elevated metabolic rates (Teleman *et al*., 2005).

The kinases SGK-1, AKT-1 and AKT-2 inactivate the forkhead transcription factor DAF-16, homolog of the mammalian FKHRL1 and the *Drosophila* FOXO, through phosphorylation that sequesters DAF-16 outside the nucleus, thereby reducing
the expression of genes involved in metabolic regulation and developmental controls (Ogg et al., 1997). Mutations in daf-16 can suppress the mutational effects of daf-2 and age-1 by bypassing the need for the IIS pathway and resulting in worms that are dauer defective and unable to enter into the dauer stage as larvae (Ogg et al., 1997).

Although many of the proteins in the core IIS pathway have been uncovered, more proteins are still being discovered such as the Drosophila insulin receptor substrate lnk (Werz et al., 2009). There is also still much more to learn about how all the components of this pathway are regulated.
2: Results

2.1 EMS Mutagenesis Screen for Novel chico Alleles

The focus of this project was to further analyze chico function by isolating new chico alleles through an ethyl methyl sulfonate (EMS) mutagenesis screen (Figure 5). Wild-type males containing a P-element with a red eye marker (w+) were exposed to 25mM EMS and crossed with white eyed amos\textsuperscript{Tb}/CyO females. From this cross the male progeny with red eyes and possibly a mutated chico locus were collected and crossed to white eyed chico\textsuperscript{1}/CyO females. Putative alleles were selected for in the F\textsubscript{2} generation based on the absence or lower than expected amount of red eyed, straight winged progeny from the complementation cross with chico\textsuperscript{1}/CyO. If there was a mutational event in the chico locus, it would be expected that the mutation over chico\textsuperscript{1} would be semi-lethal, due to the semi-lethality of chico\textsuperscript{1}/chico\textsuperscript{1}, and therefore the mutant flies would be absent or reduced in numbers (Böhni et al., 1999).
Adult males with a \( w^+ \) P-element marker (red-eye marker) inserted in an unrelated locus were exposed to 25 mM EMS and individually crossed to virgin females balanced with the \( CyO \) chromosome. In the F\(_1\) generation, red-eyed, curly winged males were selected and crossed to \( w^+:chico^1/CyO \) virgin females and scored for failure to complement.
2.2 Six Possible chico Alleles

Screening of 22,072 EMS lines produced six putative chico alleles that failed or partially failed to complement the chromosome bearing the chico1 allele. These alleles were named based on the vial number they were isolated from: chico617, chico4330, chico13063, chico13377, chico15970 and chico16906 (Table 1). The six lines were then crossed to the deficiency strain Df(2L)flp 147E to confirm if the mutation was located in the chico locus. This deficiency is a 3kb deletion created from an imprecise excision event of a P-element insertion that results in the deletion of the translation start site and regulatory region of chico and the 3′ coding sequences of basket (bsk) (Riesgo-Escovar et al., 1996; Böhni et al., 1999) (Figure 6).

![Diagram showing the location of chico and bsk with a deficiency cutting into the chico region.](image)

Figure 6: Deficiency Df(2L)flp 147E Location Relative to chico
The deficiency removes the translation start site, some of the regulatory region of chico (open boxes) and part of the bsk locus.

All putative alleles complemented the deficiency except chico13063 and chico16906. Despite the fact that we observed some interesting interactions, it was unlikely these were new chico alleles and so these lines were not pursued further for this project (Table 1).
Table 1: Cross Description Results of Putative chico Alleles

<table>
<thead>
<tr>
<th>Allele</th>
<th>Description</th>
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| chico\textsuperscript{617} | - Completely failed to complement chico\textsuperscript{1}  
  - Complemented Df(2L)\textit{flp} 147E  
  - Heterozygous chico\textsuperscript{617}/Df(2L)\textit{flp} 147E progeny normal body size |
| chico\textsuperscript{4330} | - Completely failed to complement chico\textsuperscript{1}  
  - Complemented Df(2L)\textit{flp} 147E  
  - Heterozygous chico\textsuperscript{4330}/Df(2L)\textit{flp} 147E progeny normal body size |
| chico\textsuperscript{13063} | - Partially complemented chico\textsuperscript{1}, with escaper rate of 36% (n=7,461)  
  - Partially complemented Df(2L)\textit{flp} 147E, with escaper rate of 10% (n=3,957)  
  - Heterozygous chico\textsuperscript{13063}/chico\textsuperscript{1} escapers had small body size  
  - Heterozygous chico\textsuperscript{13063}/Df(2L)\textit{flp} 147E escapers had small body size |
| chico\textsuperscript{13377} | - Partially complemented chico\textsuperscript{1}, with escaper rate of 5% (n=120)  
  - Complemented Df(2L)\textit{flp} 147E  
  - Heterozygous chico\textsuperscript{13377}/Df(2L)\textit{flp} 147E progeny normal body size |
| chico\textsuperscript{15970} | - Completely failed to complement chico\textsuperscript{1}  
  - Complemented Df(2L)\textit{flp} 147E  
  - Heterozygous chico\textsuperscript{15970}/Df(2L)\textit{flp} 147E progeny normal body size |
| chico\textsuperscript{16906} | - Partially complemented chico\textsuperscript{1}, with escaper rate of 36% (n=997)  
  - Partially complemented Df(2L)\textit{flp} 147E, with escaper rate of 28% (n=919)  
  - Heterozygous chico\textsuperscript{16906}/chico\textsuperscript{1} escapers had normal body size  
  - Heterozygous chico\textsuperscript{16906}/Df(2L)\textit{flp} 147E escapers had normal body size |

(see Appendix A for Cross Data)

Although the allele chico\textsuperscript{16906} only partially complemented the deficiency, with an escaper rate of 28\%, and therefore could still be considered a putative chico allele, there was considerable difficulty maintaining the sickly stock. Coupled with the trans-heterozygous escapers having a normal body size, further experiments with this mutant line were terminated. Trans-heterozygous escaper progeny for the allele chico\textsuperscript{13063} displayed a small body phenotype that eclosed \(~2-5\) days after other progeny from the same cross, which was also exhibited by homozygous chico\textsuperscript{1} mutants (Böhni \textit{et al.}, 1999). For this reason, the focus of the rest of this project was on the chico\textsuperscript{13063} allele.
2.3 Sequencing Results for the \textit{chico}^{13063} Allele

In order to determine if there was a nucleotide substitution that was causing the mutant phenotype the \textit{chico}^{13063} allele was sequenced. The \textit{chico}^{13063}/\textit{Df(2L)flp 147E} fly was used for sequencing and the PCR primer was designed to fall within the deletion on the deficiency chromosome, therefore, only producing PCR product from the mutant allele. A number of single nucleotide polymorphisms were found in the exons (Appendix B) as compared to the wild type sequence from Flybase (http://flybase.org/). The base pair polymorphisms in exons 1-8 did not result in a translational change of amino acids; however, there were seven base pair differences in exon 9 that did result in translational changes when compared to the Flybase amino acid reference sequence (Figure 7). When this same sequence was compared to the reference sequence of \textit{chico}^{+} given in Böhni et al.’s paper, all but one amino acid polymorphism were shown to be the same. The single amino acid difference (grey block) showed a transition from isoleucine to methionine but when the sequence was compared to the original mutagenesis chromosome sequence it was found this polymorphism was already present before EMS occurred. None of these polymorphisms map to any predicted protein domains in CHICO.
Figure 7: Amino Acid Sequence Alignment for the chico<sup>13063</sup> Allele Exon 9

The original mutagenesis chromosome sequence is shown on the top line of the alignment [chico<sup>+</sup> (mut)], followed by the chico<sup>13063</sup> allele sequence on the second line. The third line [chico<sup>+</sup> flybase] is the Flybase sequence and the fourth line [chico<sup>+</sup> (Böhni)] is the published amino acid sequence from the Böhni et al. paper. Amino acid differences are noted in shaded boxes with the differences between Flybase and all other sequences in black and the polymorphism between the reference sequences and the mutagenesis chromosome and the chico<sup>13063</sup> allele in grey. The bolded YLEM is a predicted PI3K binding site.
2.4 Size Assays for Heteroallelic Combinations of \textit{chico}^{13063} in trans with +, \textit{chico}^{1} and Df(2L)\textit{flp} 147E

Phenotypic analysis was performed on the allele to determine if it also displayed some of the characteristics the \textit{chico}^{1}/\textit{chico}^{1} mutant such as a smaller than average sized body (Böhni \textit{et al}., 1999). The \textit{chico}^{13063} line contained a dominant female sterile mutation along with the \textit{chico}^{13063} mutation, which resulted in the inability to produce homozygous \textit{chico}^{13063} flies. Homozygous \textit{chico}^{1} mutants also display a female sterile phenotype but this is in a recessive form (Böhni \textit{et al}., 1999). The \textit{chico}^{13063} line was maintained by crossing the \textit{chico}^{13063}/CyO males to white eyed \textit{chico}^{1}/CyO females and selecting balanced progeny with a linked red-eyed marker for every generation. All analysis with the \textit{chico}^{13063} allele was done in trans with +, \textit{chico}^{1} and Df(2L)\textit{flp} 147E.

2.4.1 Adult Pictures of \textit{chico}^{13063} in trans with +, \textit{chico}^{1} and Df(2L)\textit{flp} 147E

Both the female and male adult \textit{chico}^{13063}/+, \textit{chico}^{13063}/\textit{chico}^{1} and \textit{chico}^{13063}/Df(2L)\textit{flp} 147E flies appeared smaller than +/-, heterozygous \textit{chico}^{1}/+ and Df/+ as seen in Figure 8. The \textit{chico}^{13063} allele is acting in a dominant fashion and producing flies that are smaller in body size as seen in the heterozygous \textit{chico}^{13063}/+ flies compared to the +/- flies in the first column.
Figure 8: Adult Fly Size Comparison with $chico^{13063/+}$, $chico^{13063}/chico^1$ and $chico^{13063}/Df(2L)flp 147E$

The top row contains the comparison between females of $chico^{13063/+}$, $chico^{13063}/chico^1$ and $chico^{13063}/Df(2L)flp 147E$ and $+/-$, $chico^1/+$. The bottom row contains the comparison between males of $chico^{13063/+}$, $chico^{13063}/chico^1$ and $chico^{13063}/Df(2L)flp 147E$ and $+/-$, $chico^1/+$. Scale bar: 0.5mm
2.4.2 Weight Data for chico\textsuperscript{13063} in trans with +, chico\textsuperscript{1} and Df(2L)flp 147E

The size difference was quantified by weighing the adult flies (Figures 9 and 10). The adult fly weights were measured in groups of 20 and the average weight was calculated. What can be observed is that there was a significant difference in weight between chico\textsuperscript{13063}/+, chico\textsuperscript{13063}/chico\textsuperscript{1} and chico\textsuperscript{13063}/Df(2L)flp 147E flies compared to +/+ or chico\textsuperscript{1}/+ and Df(2L)flp 147E/+ flies for both females and males. The standard deviations for the wild-type or chico\textsuperscript{1}/+ and Df(2L)flp 147E/+ are wider compared to the chico\textsuperscript{13063}/+, chico\textsuperscript{13063}/chico\textsuperscript{1} and chico\textsuperscript{13063}/Df(2L)flp 147E categories, which shows the variability of these data sets. The standard deviation for the chico\textsuperscript{13063} allele in all three backgrounds is very tight around the average, which indicates the data for these sets was precise and less variable than +/+ or chico\textsuperscript{1}/+ and Df(2L)flp 147E/+. As expected, the females display an overall higher average weight compared to the males. The average weight for chico\textsuperscript{13063}/+, chico\textsuperscript{13063}/chico\textsuperscript{1} and chico\textsuperscript{13063}/Df(2L)flp 147E for females was 0.55 mg/fly and for males it was 0.45 mg/fly.
Figure 9: Average Weight Comparison for Female chico^{13065}/+, chico^{13065}/chico^{1} and chico^{13063}/Df(2L)flp 147E
Measurements of body weight were done by grouping the flies by genotype and weighing them in sets of 20 (n=3) and then calculating the average. Error bar: standard deviation of sample (*P<0.0001).
Figure 10: Average Weight Comparison for Male \textit{chico}^{13063}/+, \textit{chico}^{13063}/\textit{chico}^1 and \textit{chico}^{13063}/\text{Df(2L)flp 147E}

Measurements of body weight were done by grouping the flies by genotype and weighing them in sets of 20 (n=3) and then calculating the average. Error bar: standard deviation of sample (*$P<0.0001$).
2.4.3 Adult Wing Size Comparisons for chico^{13063/+}, chico^{13063/chico^1} and chico^{13063/Df(2L)flp 147E}

In order to confirm the size difference noted in the weight data, adult fly wings were dissected and the areas of each wing were measured. The wing size differences can be easily visualized in Figures 11 and 12. Each genotype is represented in columns; the first column is the +/+, chico^1/+ or Df(2L)flp 147E/+ wing, the second column is chico^{13063/+}, chico^{13063/chico^1} or chico^{13063/Df(2L)flp 147E} and the third column is the overlay of the two wings. In both the female and male overlays chico^{13063/+}, chico^{13063/chico^1} or chico^{13063/Df(2L)flp 147E} wings are smaller than +/+, chico^1/+ and Df/+ wings. The size difference is most noticeable in the shorter length of the wing as opposed to the width.
Figure 11: Full Wing Size Comparison for Female $chico^{13063}/+$, $chico^{13063}/chico^I$ and $chico^{13063}/Df(2L)flp 147E$

Wings were dissected from adult flies for area measurements. The first column contains $+/+$, $chico^I/+\text{ or } Df(2L)flp 147E/+$. The second column contains $chico^{13063}/+$, $chico^{13063}/chico^I$ or $chico^{13063}/Df(2L)flp 147E$. The third column is the overlay of the second column wing over the first column wing – the genotype in grey is the larger wing.
Figure 12: Full Wing Size Comparison for Male $chico^{13063}/+$, $chico^{13063}/chico^t$ and $chico^{13063}/Df(2L)flp$ 147E
Wings were dissected from adult flies for area measurements. The first column contains $+/+$, $chico^t/+$ or $Df(2L)flp$ 147E/+. The second column contains $chico^{13063}/+$, $chico^{13063}/chico^t$ or $chico^{13063}/Df(2L)flp$ 147E. The third column is the overlay of the second column wing over the first column wing – the genotype in grey is the larger wing.
The average area measurements of the dissected wings were plotted separately for females and males (Figures 13 and 16). Average area comparisons between the graphs for females and males show that the average area of the female fly wing is overall larger than the male fly wing. This would be expected considering the female fly is larger than the male. What is also seen is that the average area measurements for both the female and male wings show that the wing area for \( \text{chico}^{13063/+}, \text{chico}^{13063}/\text{chico}^1 \) and \( \text{chico}^{13063}/\text{Df}(2L)\text{flp} \ 147E \) is smaller than \(+/+, \text{chico}^1/+\) and \( \text{Df}(2L)\text{flp} \ 147E/+\). For both males and females the trend is a decrease in wing area from \( \text{chico}^{13063/+} \) being the largest to \( \text{chico}^{13063}/\text{chico}^1 \) and the smallest being \( \text{chico}^{13063}/\text{Df}(2L)\text{flp} \ 147E \). This trend in wing area is notable considering the three genotypes of the \( \text{chico}^{13063} \) allele did not show a significant difference in weight.

When the area of the wing was broken down into the length and width measurements it was clear that the length of the wing is consistently shorter for \( \text{chico}^{13063/+}, \text{chico}^{13063}/\text{chico}^1 \) and \( \text{chico}^{13063}/\text{Df}(2L)\text{flp} \ 147E \) wings compared to \(+/+, \text{chico}^1/+\) and \( \text{Df}(2L)\text{flp} \ 147E/+\) (Figures 14,15,17 and 18). The wing measurement data concurs with the previous conclusions that there is a size difference between \( \text{chico}^{13063/+}, \text{chico}^{13063}/\text{chico}^1 \) and \( \text{chico}^{13063}/\text{Df}(2L)\text{flp} \ 147E \) and \(+/+, \text{chico}^1/+\) or \( \text{Df}^+ \) and is due to a shortness in the length as opposed to width.
Figure 13: Average Wing Area Comparison for Female $chico^{15063/+}$, $chico^{15063}/chico^f$ and $chico^{13063}/Df(2L)flp 147E$

Wing areas were measured for the various genotypes and the average area was calculated (n=14). Error bar: standard deviation of sample (*$P<0.01$; **$P<0.0001$).
Figure 14: Average Length Measurements for Female \(chico^{13063}/+\), \(chico^{13063}/chico^1\) and \(chico^{13063}/Df(2L)flp\ 147E\)
Wing length were measured for the various genotypes and the average length was calculated (n=14). Error bar: standard deviation of sample (*\(P<0.0001\)).
Figure 15: Average Wing Width Measurements for Female chico^{13063}/+, chico^{13063}/chico^{f1} and chico^{13063}/Df(2L)flp 147E
Wing width were measured for the various genotypes and the average width was calculated (n=14). Error bar: standard deviation of sample.
Figure 16: Average Wing Area Comparison for Male chico\textsuperscript{15063/+}, chico\textsuperscript{15063/chico'} and chico\textsuperscript{13063/Df(2L)flp 147E}

Wing areas were measured for the various genotypes and the average area was calculated (n=14). Error bar: standard deviation of sample (*$P$<0.01; **$P$<0.0001; ***$P$<0.001).
Figure 17: Average Wing Length Measurements for Male $chico^{13063/+}$, $chico^{13063/chico^I}$ and $chico^{13063}/Df(2L)fpl 147E$
Wing lengths were measured for the various genotypes and the average length was calculated (n=14). Error bar: standard deviation of sample (*$P<0.001$; **$P<0.0001$).
Figure 18: Average Wing Width Measurements for Male *chico*{superscript 13065*/+, chico*{superscript 13065*/chico* and *chico*{superscript 13065*/Df(2L)flp 147E

Wing widths were measured for the various genotypes and the average width was calculated (n=14). Error bar: standard deviation of sample.
2.5 Eclosion Time Profile for chico\textsuperscript{13063}/+, chico\textsuperscript{13063}/chico\textsuperscript{1} and chico\textsuperscript{13063}/Df(2L)flp 147E

Along with the small size phenotype, chico\textsuperscript{1}/chico\textsuperscript{1} flies also eclose 2-3 days after their heterozygous siblings (Böhni et al., 1999). In order to determine if the chico\textsuperscript{13063} allele also displayed these characteristics an eclosion profile was made (Figures 19-24). The daily eclosion of chico\textsuperscript{13063}/+, chico\textsuperscript{13063}/chico\textsuperscript{1} and chico\textsuperscript{13063}/Df(2L)flp 147E was plotted against the percent total eclosion with the genders analyzed separately. There is a significant time difference of 3-5 days between the eclosion of +/+, +/CyO, chico\textsuperscript{1}/+ and Df(2L)flp 147E/+ as compared to chico\textsuperscript{13063}/+, chico\textsuperscript{13063}/CyO, chico\textsuperscript{13063}/chico\textsuperscript{1} and chico\textsuperscript{13063}/Df(2L)flp 147E. Notably, the later eclosion is not gender specific as there is not a significant difference between female and male eclosion times. There is a slight difference in eclosion between chico\textsuperscript{13063}/CyO and chico\textsuperscript{13063}/chico\textsuperscript{1} or chico\textsuperscript{13063}/Df(2L)flp 147E, with chico\textsuperscript{13063}/chico\textsuperscript{1} and chico\textsuperscript{13063}/Df(2L)flp 147E showing a slight delay of approximately one day in eclosion as compared to chico\textsuperscript{13063}/CyO.
Figure 19: Eclosion Profile for Female \textit{chico}^{13065/+}

Figure 20: Eclosion Profile for Female \textit{chico}^{13065}/chico'}
Figure 21: Eclosion Profile for Female chico^{1,506}/Df

Figure 22: Eclosion Profile for Male chico^{1,506}/+
Figure 23: Eclosion Profile for Male $chico^{1s065}/chico^1$

Figure 24: Eclosion Profile for Male $chico^{1s065}/Df$
3: Discussion

In this thesis, I set out to investigate the relationship between the structure and function of chico in the IIS pathway by creating novel chico alleles. When the insulin receptor substrate chico was characterized by Böhn et al. over a decade ago, they predicted the protein sequence to contain a number of binding domains that were important for its function. These domains include, in the N-terminal region, a PH and a PTB domain and, in the C-terminal region, several SH2-binding motifs. Although some of the structure of chico has been predicted, the significance of the structure to the function of chico has not been well established. In order to examine the dynamics between structure and function, the idea was to create novel chico alleles using an EMS screen and then ascertain where in the sequence the mutation occurred. By mutating the sequence, the importance of the different domains of chico could be determined in relation to its function in the IIS pathway.

EMS Screen for Novel chico Alleles

An EMS mutagenesis screen (Figure 5) was used to produce the new alleles because EMS, under optimized conditions, produces single base pair mutations that result in a difference in the amino acid sequence compared to a P-element screen that are more likely to produce complete disruptions in the gene (Sullivan et al., 2000). The adult male flies from the EMS cross that potentially contained mutations in the chico locus were crossed to chico¹/CyO virgins to test if any of the EMS mutants would fail to complement
This allowed for the screen to be fairly efficient in the processing of the mutagenized lines because a failure to complement chico\textsuperscript{1} would produce vials with very little to no straight winged flies, which would be easily screened. This screen was designed to select for mutations in chico that produced low to no complementation of chico\textsuperscript{1} due to chico\textsuperscript{1}/chico\textsuperscript{1} being semi-lethal (Böhni et al., 1999). At the beginning of this project, the only available allele of chico was chico\textsuperscript{1}, which was the result of a P-element insertion 80 base pairs downstream of the translational start site and therefore was predicted to be a null allele (Böhni et al., 1999). The selection of mutants that failed to complement the chico\textsuperscript{1} semi-lethality resulted in a screen that was biased toward strong hypomorphic and null alleles such as mutations occurring in the regulatory region of the gene. It is possible that weaker alleles affecting certain functional domains would have had visible phenotypes but no reduced viability and these may not have been detected using our screening approach. Since the completion of the mutagenesis screen a new IRS protein, lnk, was found to act in parallel with chico in the IIS pathway (Werz et al., 2009). This gene shows a dominant genetic interaction with chico, therefore, an improvement to the screen could have been the use of a weaker lnk allele in the complementation cross and then observing the progeny for an enhancement of the IRS mutant phenotypes.

**Putative chico Alleles**

After screening 22,072 EMS lines, six putative chico alleles were produced that either completely failed to complement chico\textsuperscript{1} or only allowed for a low percentage of escapers. These six alleles were chico\textsuperscript{617}, chico\textsuperscript{4330}, chico\textsuperscript{13063}, chico\textsuperscript{13377}, chico\textsuperscript{15907} and
Each of these alleles were then crossed to the deficiency strain $Df(2L) 147E$ (Figure 6) that is a deletion of the translation start site and regulatory region of $chico$. All but $chico^{13063}$ and $chico^{16906}$ complemented the deficiency $Df(2L)flp 147E$ and although there were some interesting interactions, it was likely that these alleles were not $chico$ alleles and were disregarded for future analysis in this project. It is interesting that these mutagenized chromosomes failed to complement $chico^l$. These chromosomes may have carried second site mutations on the second chromosome in other genes that interact with $chico$ in the IIS pathway such as the subunit of $PI3K$, $p60$. However, they could not have been alleles of $lnk$ because this gene is on the third chromosome, a chromosome not balanced in the original screen. It is also a possibility that the original $chico^l$ chromosome was carrying some recessive mutations that when crossed with the mutated chromosome from the various putative alleles, resulted in non-complementation that was not due to a mutation in the $chico$ gene.

Both $chico^{13063}$ and $chico^{16906}$ alleles were different from the other alleles in that they partially complemented $chico^l$ with a lower than expected amount of escapers, 36% for both alleles. They also partially complemented the deficiency $Df(2L)flp 147E$, with escaper rates of 10% for $chico^{13063}$ and 28% for $chico^{16906}$, which indicated they could both still be considered putative alleles of $chico$. The difference between the two alleles were that the heterozygous escapers $chico^{13063}/chico^l$ and $chico^{13063}/Df (2L)flp 147E$ had a small body phenotype characteristic of $chico$ mutants and the heterozygous escapers $chico^{16906}/chico^l$ and $chico^{16906}/Df(2L)flp 147E$ did not. Along with the extreme difficulty of maintaining the $chico^{16906}$, the decision was made to focus the study on the allele $chico^{13063}$ and to not continue with further experiments on the $chico^{16906}$ stock. It is
a possibility that \textit{chico}^{16906} was an allele of \textit{chico} that was not as strong of an allele as \textit{chico}^1 or the heterozygous \textit{chico}^{13063} and that is why \textit{chico}^{16906}/\textit{chico}^1 and \textit{chico}^{16906}/\text{Df(2L)flp 147E} flies did not have the small body phenotype. In order to determine if \textit{chico}^{16906} allele was a bona fide allele of \textit{chico} a rescue assay could have been performed using a construct expressing a wild-type copy of \textit{chico} in the \textit{chico}^{16906} mutant. Given that \textit{chico}^{13063} homozygous flies could not be recovered, we could have tested whether expression of \textit{chico} using the Gal4-UAS system could have rescued the dominant female sterile phenotype and allowed recovery of homozygous flies (Brand and Perrimon, 1993).

**The \textit{chico}^{13063} Allele**

Phenotypic analysis of \textit{chico}^{13063} was done in trans with \textit{chico}^1 and \text{Df(2L)flp 147E} because the \textit{chico}^{13063} line contained a dominant female sterile mutation and homozygous mutants could not be obtained. The female sterility mutation is a characteristic of the partial loss-of-function mutation \textit{dimr}^{E19} in the INR and the null mutation \textit{chico}^1 in the IRS (Brogiolo \textit{et al.}, 2001; Böhni \textit{et al.}, 1999). This common phenotype would support \textit{chico}^{13063} as an allele of \textit{chico} and that the female sterile mutation is not the result of a separate mutational event but of a phenotype displayed by \textit{chico} mutants. What is unexpected of the female sterile phenotype is that it is dominant in the \textit{chico}^{13063}/+ mutant and recessive in the \textit{dimr}^{E19} and \textit{chico}^1 mutants. This mirrors the growth phenotype as well, which is dominant in our new \textit{chico}^{13063} allele yet recessive in the existing \textit{chico}^1 allele.
The coding region of the chico\textsuperscript{13063} allele was sequenced and a number of base pair polymorphisms were found when the sequence was compared to the chico\textsuperscript{+} sequence from Flybase. These polymorphisms were present in the mutagenesis allele and when translated did not result in changes to the amino acid sequence in exons 1-8 (Figure 7). There were seven polymorphisms that did result in changes to the translated sequence in exon 9, a part of the C-terminal phosphorylation domain of chico, when compared to the sequence from Flybase. When the amino acid sequence translated from chico\textsuperscript{13063} was compared to the sequences published by Böhni et al. and Kulansky Poltilove et al., it was found that they were almost identical. The one difference between these two translations was a transition from isoleucine to methionine in exon 9, which was present in the mutagenesis chromosome, indicating another polymorphism not previously identified is present in the chico sequence (Figure 7). None of the polymorphisms occur in the predicted SH2 binding sites for the C-terminus end of CHICO. The mutation causing the chico phenotype could be located upstream of the coding sequence and likely disrupting some part of the regulatory region. The mutant chico\textsuperscript{13063} displays the classic IRS phenotypic characteristic of small body size that even as a heterozygote, the chico\textsuperscript{13063} phenotype is stronger than chico\textsuperscript{1/+} and Df(2L)flp 147E/+. The mutation in the chico\textsuperscript{13063} allele could be acting in a dominant negative way in the IIS pathway and in some way be interfering with the wild-type copy of the gene, although the absence of a mutation in the coding sequence seems to preclude such an interaction. It is also possible that it is interacting with the other IRS protein lnk, which could cause a down regulation in the IIS pathway. In order to more fully understand the nature of the defect in our chico
allele, future analyses should investigate expression levels of CHICO in the 
\(chico^{13063}\) mutant to assess whether regulatory mutations have occurred.

**Size Assays for the \(chico^{13063}\) Allele**

The small body phenotype is characteristic of mutations in the INR and IRS of *Drosophila* (Brogiolo *et al*., 1995; Böhni *et al*., 1999). Comparison of \(chico^{13063}/+\) adults to the control, \(chico^+/+\) and \(Df(2L)flp\ 147E/+\) adults quantitatively demonstrated that both males and females were visibly smaller for the mutant \(chico^{13063}\). The overall growth of the fly can be largely affected by two factors: hormones/growth factors and nutrient availability. The growth of *Drosophila* under unfavorable food conditions yields small flies with fewer and smaller cells (Hietakangas and Cohen, 2009). The flies for all the size assays were grown under lower density conditions with a sufficient amount of food to minimize the impact of nutrient amounts on growth, thereby ensuring the small body phenotype was due to the mutation and not environmental factors.

Quantitative results of the size difference for \(chico^{13063}/+\), \(chico^{13063}/chico^+\) and \(chico^{13063}/Df(2L)flp\ 147E\) included weight measurements of the adult flies and comparison of the average weight per fly (Figures 9 and 10). The difference in weight for the female flies is more obvious than the males with an average difference of 0.42 mg/fly between the mutants \(chico^{13063}/+\), \(chico^{13063}/chico^+\) and \(chico^{13063}/Df(2L)flp\ 147E\) and the control or \(chico^+/+\) and \(Df(2L)flp\ 147E/+\). The males had an average difference of 0.27 mg/fly between the mutants \(chico^{13063}/+\), \(chico^{13063}/chico^+\) and \(chico^{13063}/Df(2L)flp\ 147E\) and the control or \(chico^+/+\) and \(Df(2L)flp\ 147E/+\). The larger difference in weights for the females was expected considering the mutants \(chico^{13063}/+\),
chico^{13063}/chico^1 and chico^{13063}/Df(2L)flp 147E females were sterile, which most likely meant that the oocytes failed to mature and develop eggs. Homozygous chico^1 female sterility is caused by their oocytes not maturing past the last previtellogenic stage (Richard et al., 2005). The average weight of the chico^{13063}/+, chico^{13063}/chico^1 and chico^{13063}/Df(2L)flp 147E flies are within a tight range for both females (0.42-0.54 mg/fly) and males (0.44-0.45 mg/fly). The standard deviation error bars for the female and male chico^{13063}/+, chico^{13063}/chico^1 and chico^{13063}/Df(2L)flp 147E mutants demonstrate how tight the data variation is for the mutant as compared to the wild-type control or chico^1/+ and Df(2L)flp 147E/+. This means that the size of the chico^{13063}/+, chico^{13063}/chico^1 and chico^{13063}/Df(2L)flp 147E flies were not limited by variable environmental factors but rather reached the limit of genetic expression. Although chico^1/chico^1 flies were shown to be dramatically reduced in weight compared to wild-type flies, this is a recessive phenotype for chico^1 mutants. Heterozygous chico^1/+ flies were slightly larger in weight when compared to wild-type flies (Böhni et al., 1999). We see that not only does chico^{13063}/chico^1 and chico^{13063}/Df(2L)flp 147E have reduced weight but that chico^{13063}/+ does also; therefore, indicating that the chico^{13063} allele is functioning in dominant negative manner.

Further investigation of the smaller size was performed through the analysis of the wing areas of chico^{13063}/+, chico^{13063}/chico^1 and chico^{13063}/Df(2L)flp 147E, which was seen in the overlays of the adult wings (Figures 11 and 12), as well as, wing area measurements for both females and males (Figures 13 and 16). Visual comparison of the wing sizes showed chico^{13063}/+, chico^{13063}/chico^1 and chico^{13063}/Df(2L)flp 147E wings were smaller than the wild-type control or chico^1/+ and Df(2L)flp 147E/+ with the
difference in size most visibly seen as shorter wing length. Area measurements of the entire wing confirmed that the size of the mutant wing was smaller and length and width measurements further verified that the difference in area measurements was due mostly to the shortness of the wing length rather than a difference in width (Figures 14, 15, 17 and 18).

When wing area measurements performed for the allele chico1 by Böhni et al. chico1/+ had a 4.8% reduction in wing size and chico1/chico1 had a 39.5% size reduction when compared to wild-type wings. chico13063/+ had an average reduction in wing area of 5.2% when compared to wild-type wings and an average reduction of 18.9% for chico13063/chico1 compared to +/+ and 25.6% for chico13063/Df(2L)flp 147E compared to +/+. We do see a reduction of wing size for the chico13063 allele, but in the heterozygous form of chico13063/+ it is acting like chico1/+ with a comparable percentage reduction in size. When chico13063/chico1 and chico13063/Df(2L)flp 147E size reductions are compared to that of chico1/chico1 the reduction is not as large as the 39.5% reduction but is more than chico13063/+, which is consistent with the data presented by Böhni et al. It is very likely that we would observe a more dramatic reduction in wing size if we were to recover chico13063 homozygous adults.

Eclosion Assay for the chico13063 Allele

Another characteristic phenotype of chico1 mutants is a delay in development with chico1/chico1 mutants eclosing 2-3 days after their heterozygous siblings (Böhni et al., 1999). The eclosion profiles of chico13063/+, chico13063/chico1 and chico13063/Df(2L)flp 147E for both females and males showed that this mutation is
consistent with the \textit{chico}^{I}/\textit{chico}^{I} mutation with \textit{chico}^{13063/+} beginning to eclose two days after the wild-type control flies, as well as, \textit{chico}^{13063}/CyO eclosing two days after \textit{chico}^{I/+} or \textit{Df(2L)flp 147E/+} (Figures 19-24). There is a slight delay of \textit{chico}^{13063}/\textit{chico}^{I} or \textit{chico}^{13063}/Df(2L)flp 147E egression of approximately a day as compared to \textit{chico}^{13063}/CyO, showing the mutant in trans to \textit{chico}^{I} and \textit{Df(2L)flp 147E} is slightly worse than a single copy of the mutant allele.

**Future Analysis of the \textit{chico}^{13063} Allele**

If the mutation in the \textit{chico}^{13063} allele is present in the regulator region and is affecting the expression of the gene in some way the next step in the analysis of the \textit{chico}^{13063} allele would be to look at the expression levels of CHICO. This could be done by quantifying the levels of CHICO mRNA in the mutant using techniques such as Northern blotting (Kevil \textit{et al}., 1997) or quantitative reverse transcription polymerase chain reaction (qPCR) (Nolan \textit{et al}., 2006).

Another critical next step in the analysis of the \textit{chico}^{13063} allele would be to perform a rescue assay for the mutant phenotype by expression of a wild-type construct of \textit{chico} in a \textit{chico}^{13063/+} fly by using the Gal4-UAS system (Jäckle \textit{et al}., 1986; Brand and Perrimon, 1993). Rescuing the mutant phenotype could require rescuing the female sterile mutation and would prove therefore that this is a phenotype of the \textit{chico}^{13063} allele versus the result of a separate mutational event.

It has been established that the \textit{chico}^{13063} allele produces flies that are smaller in body size and wing size; however, it is unclear based on these results whether this decrease in size can be attributed to a reduction in cell size and cell number as seen in
INR and IRS mutants (Brogiolo et al., 2001; Böhni et al., 1999). One way this could be
determined would be to measure the cell density in the wing. As each cell in the wing
epithelium gives rise to a single wing hair, the density could be assessed by counting the
number of wing hairs in a set area of the wing. When the whole adult wings were
dissected and measured for total wing area (Figures 13 and 16), magnified pictures of the
wing surface and hairs were also taken of the area just posterior to the posterior cross
vein. The next step would be to count the number of wing hairs to determine the density
and if the size reduction is due to a reduction in cell size, cell number or both.

c\textsuperscript{13063}chico\textsuperscript{1}/chico\textsuperscript{1} mutants showed an overall reduction in size for all developmental stages
from embryo to adult fly (Böhni et al., 1999). Although obtaining homozygous chico\textsuperscript{13063}
mutants is not possible, observing the developmental stages of chico\textsuperscript{13063}/+,
chico\textsuperscript{13063}/chico\textsuperscript{1} and chico\textsuperscript{13063}/Df(2L)flp 147E could be possible. Even though INR and
IRS mutants are smaller in size, both dinr\textsuperscript{E19} (an allele of INR) and chico\textsuperscript{1} mutants have
an almost 2-fold increase in lipid content (Brogiolo et al., 2001; Böhni et al., 1999). A
further test to characterize the chico\textsuperscript{13063} allele would be to test the lipid content of the fly
tissue and compare the lipid content to INR and IRS mutants.

In *C. elegans*, mutations in certain insulin signalling genes, specifically the insulin
receptor *daf-2* and the PI3K subunit *age-1* result in an increase of adult life-span by up to
200% (Guarente and Kenyon, 2000). Clancy et al. tested genes known to be a part of the
IIS pathway in *Drosophila: Inr, chico*, subunits of PI3K (*p110* and *p60*) and *Akt1*, and
found that only mutations in *chico* produced progeny with extended life-span by up to
48% for homozygotes and 36% for heterozygotes. Another phenotypic analysis for the
chico\textsuperscript{13063} allele would be to test and see if chico\textsuperscript{13063}/+, chico\textsuperscript{13063}/chico\textsuperscript{1} and chico\textsuperscript{13063}/Df(2L)flp 147E display an increase in lifespan characteristic of chico mutants.

Conclusion

The EMS mutagenesis screen for novel chico alleles did produce one putative allele, chico\textsuperscript{13063}. The sequencing of the chico\textsuperscript{13063} allele demonstrated a number of base pair polymorphisms that are present in the chico sequence but did not show any mutations in the coding region leading to the hypothesis that the mutation exists in the regulatory region of chico. Therefore, investigation of the structure as it relates to the function of chico was not deduced from the chico\textsuperscript{13063} allele due to the mutation not falling within the coding region. Phenotypic analysis of chico\textsuperscript{13063} supports the identity of this allele as a putative allele of chico by establishing that this novel allele produces the characteristic phenotypes demonstrated by Böhni et al. of the small body and small wing, female sterility, and delayed development showed by the late eclosion of the heterozygous chico\textsuperscript{13063} adults compared to the heterozygous siblings. Future experiments for chico\textsuperscript{13063} would include a rescue assay, determining expression levels of CHICO, sequencing the regulatory regions, determining if the small size is due to a decrease in cell number, cell size or both, examining ovary development, and looking at if there is life-span extension.
4: Materials and Methods

4.1 Drosophila Handling

Flies were maintained on a standard cornmeal/yeast/agar media (see Appendix E). All stocks and crosses were maintained at 25°C unless otherwise noted.

4.2 Drosophila Stocks

The following stocks were obtained from the Bloomington Stock Center:

\( cn^1 P\{ry11\}chico^1/CyO; ry^{506} \) (Böhni et al., 1999) – referred to as \( chico^1/CyO \) in this text

\( amos^{T0}/CyO \)

\( \{b P\{lac w^+\}\} \) insert into 5’ UTR of Gliotactin (Auld et al., 1995) – the original mutagenesis stock used as an eye colour marker

\( Df(2L)flp147E/CyO \) (Goberdhan et al., 1999; Böhni et al., 1999) – was a gift from Esther Verheyen.

4.3 EMS Mutagenesis Screen

This EMS procedure was adapted from Drosophila Protocols (Sullivan et al., 2000). Approximately 20-40 adult males were fed 25mM EMS (Sigma-Aldrich) solution (0.13 ml of EMS in 4% sucrose solution) by dispensing 1.1ml of EMS/sucrose solution on 2x filter paper fitted to the bottom of glass jars. Males with the red eye and black body markers \( \{b P\{lac w^+\}\} \) were used as the wild-type stock. The males were left overnight on the EMS solution and turned over onto fresh food the following day. Virgin
amos\textsuperscript{Th}/CyO females were introduced to the males at least three hours after the males were removed from EMS (or up to 24 hours later). The males and females were then separated after 72 hours (maximum); the males were destroyed and the females were put onto fresh food. Virgin amos\textsuperscript{Th}/CyO and chico\textsuperscript{1}/CyO females were created for this screen using a heat-shock induced conditional lethal transgene inserted on the Y chromosome of male flies (Starz-Gaiano et al., 2001; Venema, 2006). Bottles of flies were heat-shocked for two hours at 37°C in a water bath to kill the males.

4.4 Isolating Genomic DNA

This genomic DNA preparation method was adapted from Drosophila Protocols (Sullivan et al., 2000). Approximately 30 chico\textsuperscript{13063/Df(2L)flp 147E flies were collected into a 1.5 ml microcentrifuge tube and either frozen at -80°C or ground with 400 µl of buffer A (100mM Tris-Cl at pH 7.5, 100mM EDTA, 100 mM NaCl, 0.5% SDS) and a sterilized tissue grinder. The ground tissue in buffer A was incubated at 65°C for 30 minutes followed by the addition of 800 µl of buffer B (200 ml of 5M potassium acetate and 500 ml of 6M lithium chloride stored at 4°C). The sample was incubated on ice for approximately one hour and then centrifuged at 12,000 rpm for 15 minutes (at room temperature). Supernatant from the sample was transferred to a new microcentrifuge tube and 600 µl of isopropanol was added to the supernatant and inverted. The tube was then centrifuged at 12,000 rpm for 15 minutes (at room temperature) and the supernatant was discarded. The pellet was washed with 70% ethanol and air dried after which it was re-suspended in 150 µl of TE buffer (10mM Tris-Cl at pH 7.4 and 1mM EDTA at pH8). The sample was stored at -20°C until needed.
4.5 PCR of chico\textsuperscript{13063} from Genomic DNA

PCR primers were designed for the full length Drosophila genome sequence 2L:10242512..10247064 of chico using the program Primer3 (v.0.4.0) (http://frodo.wi.mit.edu/primer3/) (Table 2). The forward primer anneals to the template 81 base pairs upstream of the first exon of chico. The reverse primer anneals to the template 129 base pairs after the last exon. Tm calculations were performed using the nearest neighbour method (Breslauer et al., 1986; http://www.finnzymes.com).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chico-reverse</td>
<td>CAGGATTTTCGGACAAGAGA</td>
<td>62.3 °C</td>
</tr>
<tr>
<td>Chico-forward</td>
<td>AATAAGCCGGCGTGTAATAC</td>
<td>63.0 °C</td>
</tr>
</tbody>
</table>

The primers were ordered from Invitrogen and re-suspended in ddH\textsubscript{2}O to make a primer concentration of 50 µM stock solution. The primers were further diluted to 20µM to create a working stock for PCR. Both stock solutions were stored at -20°C.

The PCR reaction produced a 4.5kb fragment that included the exons and introns of chico\textsuperscript{13063} using the high-fidelity DNA polymerase Phusion (Pfu) in order to reduce possible sequencing errors (Finnzymes, New England BioLabs). Reagent concentrations for the PCR reaction followed the Pfu DNA Polymerase guidelines for a 50µl reaction: 10µl of 5x Pfu HF buffer (1.5mM MgCl\textsubscript{2} in final reaction), 1.5µl of 10mM dNTPs, 2.5µl (2x) of 20µM primer (for a final concentration of 0.5µM), 0.5µl of 2U/µl Pfu DNA Polymerase, 1µl of genomic DNA template (with a concentration range of 50-100ng/µl), and 31.5µl ddH\textsubscript{2}O. The PCR reactions were performed in a BioRad MyCycler using the
3-step protocol recommended for the *Pfu* enzyme (Table 3). The PCR product was then purified using a QIAquick PCR purification kit (Quiagen) and stored at -20°C.

<table>
<thead>
<tr>
<th>Cycle Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
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<td>40 seconds</td>
<td>1x</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98.0°C</td>
<td>10 seconds</td>
<td>35x</td>
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<tr>
<td>Annealing</td>
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<tr>
<td>Extension</td>
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<td>4 minutes</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72.0°C</td>
<td>10 minutes hold</td>
<td>1x</td>
</tr>
<tr>
<td></td>
<td>4.0°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 4.6 Cloning of *chico*<sup>13063</sup> PCR Product

*Pfu* DNA polymerase generated a blunt ended 4.5kb PCR product of *chico*<sup>13063</sup> that needed to be modified before inserting into the plasmid for cloning. The protocol from *Promega Notes* was used to add non-template dependent A-residues to the 3’ end of the blunt ended PCR fragment (Knoche and Kephart, 1999). The maximum starting amount of 7µl of purified PCR product was used and 1µl *Taq* DNA Polymerase 10x reaction buffer with MgCl₂, 0.2µl of dATP (0.2mM final concentration), 1µl (5 units) of *Taq* DNA Polymerase and 0.8µl of ddH₂O were added to bring the total volume to 10µl. The reaction mixture was incubated at 70°C for 30 minutes in a BioRad MyCycler.

The modified 4.5kb fragment was ligated into the vector pGEM<sup>®</sup> using the pGEM<sup>®</sup>-T Easy Vector System (Promega Corporation). The volumes for the reaction were as follows: 5µl 2x Rapid Ligation Buffer (T4 DNA Ligase), 1µl pGEM<sup>®</sup>-T Easy Vector, 3.125µl PCR product, 1µl T4 DNA ligase for a total reaction volume of 10µl. A positive and background control were also made containing a control DNA insert and no
DNA insert respectively. The reactions were incubated overnight at 4°C and then transformed into DH5α competent cells. The DH5α competent cells were prepared using a standard protocol (Inoue et al., 1990), dispensed into 1ml aliquots and stored at -80°C. Transformation of the cells began with thawing the cells at room temperature and dispersing 200µl aliquots into 15ml polypropylene tubes that were sitting in an ice bath. 5µl of transformant plasmid was added to each tube and incubated in the ice bath for 30 minutes. The cells were then heat-pulsed without agitation at 42°C for 30 seconds and then transferred back to the ice bath. 0.8ml of SOC (super optimal catabolite repression media) was then added to the cells and incubated at 37°C and shaken at 200rpm for 1 hour (Inoue et al., 1990). 25µl of transformation culture were then plated into duplicate LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C. Positive colonies were isolated from the plate and grown overnight in LB/ampicillin broth and maintained on LB/ampicillin plates.

### 4.7 Sequencing chico\textsuperscript{13063}

The sequencing of chico\textsuperscript{13063} in either the plasmid or PCR form was carried out through the single extension protocol offered at Macrogen Inc. (http://www.macrogen.com). The full 4.5kb fragment was sent with internal primers that included both the introns and exons (Table 4). The sample was sent in a concentration of approximately 100ng/µl for plasmid samples and 50ng/µl for PCR product. An average base pair read of 500 was guaranteed by Macrogen; therefore, primers were designed every 300 base pairs and the primer pairs were designed to overlap by at least 50 base pairs to allow for the increased error frequency at the beginning and end of sequencing reads (Figure 25).
Table 4: Internal PCR Primers for the Sequencing of *chico*\textsuperscript{13063}

<table>
<thead>
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<th>Name</th>
<th>Sequence</th>
<th>Tm</th>
<th>Size of Product</th>
</tr>
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<tr>
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<tr>
<td>chcoL2S</td>
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<tr>
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<tr>
<td>chcoR3S</td>
<td>ACGGCGATTGAGGAATCTGAAG</td>
<td>64.1°C</td>
<td></td>
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<tr>
<td>chcoL4S</td>
<td>GCCAAAAAGGGTTATATATCTGAAG</td>
<td>62.0°C</td>
<td>488bp</td>
</tr>
<tr>
<td>chcoR4S</td>
<td>ACAATCCCTGGATTTTGCA</td>
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<tr>
<td>chcoL5S</td>
<td>GTGGTTGCAAGCAAAACTGC</td>
<td>66.0°C</td>
<td>480bp</td>
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<tr>
<td>chcoR5S</td>
<td>ACTGCAGCTGCGCAATTC</td>
<td>65.9°C</td>
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<td>chcoL6S</td>
<td>AGTCAAAAACCTCTCTGAATACG</td>
<td>61.8°C</td>
<td>500bp</td>
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<tr>
<td>chcoR6S</td>
<td>TGCTAAATAATTCCGCACTGG</td>
<td>64.0°C</td>
<td></td>
</tr>
<tr>
<td>chcoL7S</td>
<td>ATGGATGACTTTTGGGACTGC</td>
<td>63.2°C</td>
<td>485bp</td>
</tr>
<tr>
<td>chcoR7S</td>
<td>GATTGGAGCCAAACCGAGTA</td>
<td>64.3°C</td>
<td></td>
</tr>
<tr>
<td>chcoL8S</td>
<td>GTGCAGCTACTGGTTGGAATCTGC</td>
<td>66.3°C</td>
<td>480bp</td>
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<tr>
<td>chcoR8S</td>
<td>GCAATTCTAAACTGAGGAGGATGTG</td>
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<tr>
<td>chcoL9S</td>
<td>ATGAAGGCCAGTGTTTGGGATAAATGG</td>
<td>64.2°C</td>
<td>490bp</td>
</tr>
<tr>
<td>chcoR9S</td>
<td>TGTAGCCGGCTTGGTTAATAT</td>
<td>63.7°C</td>
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</tr>
<tr>
<td>chcoL10S</td>
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<td>500bp</td>
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<tr>
<td>chcoR10S</td>
<td>GAGGAGCTGGATTTTGCTAAA</td>
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<td>chcoL11S</td>
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4.8 Weight Measurements

Adult flies were collected from crosses every two days after the first day of eclosion and frozen at -80°C until ready to weigh. The flies were thawed for one hour and weighed on a Mettler Toledo AB104-S/FACT (range: 0.01-110 g) scale in groups of 20 flies (n=3) and the average weight per fly was calculated.

4.9 Adult Wing Dissections

Both wings were dissected from adult flies, dehydrated in ethanol, washed twice and mounted with DL-Lactic acid, 85% w/w (Sigma).

4.10 Adult Wing Size Measurements

The dissected adult wings were photographed using a Nikon Eclipse E600 microscope and the 4x objective lens with a Leica DFC320 digital camera. The images were captured using the Leica Application Suite (version 2.5.0 R1) as JPEG files and analyzed in Photoshop CS4. The wing area was measured by first aligning the posterior crossvein vertically and then drawing around the perimeter of the wing. The cut off points on the anterior portion of the wing was from the inside edge of the posterior end of the alar lobe and the posterior tip of the distal costa (Figure 26).
Figure 26: Adult Wing Size Measurements
   Red line shows the proximal cut off point for the size measurements of the adult wing

   The measurements of the selected portion of the wing was recorded in bits and then converted to millimetres by comparing these measurements with a photograph of a 2mm scale micrometer.

4.11 Eclosion Timeline Measurement

   Adult flies were collected every 24 hours from the first day of eclosion for a total of ten days and frozen at -80°C until they could be sorted and counted. Statistical analysis was performed using PASW Statistics 17.0 and the data was graphed as Kaplan Meier plots.
5: Reference List


Appendix A – Cross Data Showing Reduced Viability of novel chico alleles, chico$^{13063}$ and chico$^{16906}$, in trans with chico$^1$ and Df(2L)flp 147E

Table 5: Cross Data for w$^{-}$; chico$^{13063}$/CyO x w$^{-}$; chico$^1$/CyO

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Total</th>
<th>% Total</th>
<th>% Normalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>w$^{-}$; chico$^1$/CyO</td>
<td>White eye, curly wing</td>
<td>4316</td>
<td>58</td>
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<td>w$^{-}$; chico$^{13063}$/CyO</td>
<td>Red eye, curly wing</td>
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<td>w$^{-}$; chico$^{13063}$/chico$^1$</td>
<td>Red eye, straight wing</td>
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<td>15</td>
<td>36</td>
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<tr>
<td>Total</td>
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<td>7461</td>
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</table>

Table 6: Cross Data for w$^{-}$; chico$^{13063}$/CyO x w$^{-}$; Df(2L)flp 147E/CyO

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Total</th>
<th>% Total</th>
<th>% Normalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>w$^{-}$; Df/CyO</td>
<td>White eye, curly wing</td>
<td>2331</td>
<td>59</td>
<td></td>
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<tr>
<td>w$^{-}$; chico$^{13063}$/CyO</td>
<td>Red eye, curly wing</td>
<td>1431</td>
<td>36</td>
<td></td>
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<tr>
<td>w$^{-}$; chico$^{13063}$/chico$^1$</td>
<td>Red eye, straight wing</td>
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<td>10</td>
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<tr>
<td>Total</td>
<td></td>
<td>3957</td>
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</table>

Table 7: Cross Data for w$^{-}$; chico$^{16906}$/CyO x w$^{-}$; chico$^1$/CyO

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Total</th>
<th>% Total</th>
<th>% Normalized</th>
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</thead>
<tbody>
<tr>
<td>w$^{-}$; chico$^1$/CyO</td>
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<td>541</td>
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<td>w$^{-}$; chico$^{16906}$/CyO</td>
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<td>Red eye, straight wing</td>
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<tr>
<td>Total</td>
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<td>997</td>
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</table>

Table 8: Cross Data for w$^{-}$; chico$^{16906}$/CyO x w$^{-}$; Df(2L)flp 147E/CyO

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Total</th>
<th>% Total</th>
<th>% Normalized</th>
</tr>
</thead>
<tbody>
<tr>
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<td>White eye, curly wing</td>
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</tr>
<tr>
<td>w$^{-}$; chico$^{13063}$/CyO</td>
<td>Red eye, curly wing</td>
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</tr>
<tr>
<td>w$^{-}$; chico$^{13063}$/chico$^1$</td>
<td>Red eye, straight wing</td>
<td>12</td>
<td>12</td>
<td>28</td>
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<tr>
<td>Total</td>
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<td>100</td>
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</tbody>
</table>
Appendix B – Coding Sequence Alignment of chico\textsuperscript{13063} and Mutagenesis Chromosome

Exon 1

```
chico'2003
chico
chico
chico
chico
chico
chico
chico
chico
chico
Exon 1
```

Exon 2

```
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
Exon 2
```

Exon 3

```
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
Exon 3
```

Exon 4

```
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chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
Exon 4
```

Exon 5

```
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chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
chico'2003
Exon 5
```

chic013063

Exon 6
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chico'
chica' chico'
chico'
chica' chico'
chico'
chica' chico' chico'

Exon 7
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chico'
chico'

Exon 8
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chico' chico'
chico' chico' chico' chico'

gt ctc ggc aag gag aag tgc gat agc tta cca acc aga aat gga acc cta agc
gt ttc ggc aag gag aag tgc gat agc tta cca acc aga aat gga acc cta agc
gag tcc agc aat cca acc cta ttt ggt tcc acc cat gga ctc cga tcc aat act
gag tcc agc aat cca acc cta ttt ggt tcc acc cat gga ctc cga tcc aat act
ata tct ggc aca cgt ccc cac tca acc aac aag cat agt aat agt cca aag tcc
ata tct ggc aca cgt ccc cac tca acc aac aag cat agt aat agt cca aag tcc
acc agt cca tta aga tgc tca gaa tcc gaa gag tca tca att aat gtc gat gaa
acc agt cca tta aga tgc tca gaa tcc gaa gag tca tca att aat gtc gat gaa
tcc gac gac acc ggc aag ttt agc cac tac ags tta aag
tcc gac gac acc ggc aag ttt agc cac tac ags tta aag

c cgc ggg tca ttc gag aag gca att cct gag gaa aac att gat gac ttt ggc
c cgc ggg tca ttc gag aag gca att cct gag gaa aac att gat gac ttt ggc
aat ccc cca tta ttt aac aaa gtc acc cca cca aat cc
gat gcg gaa tta ttt aac aaa gtc acc gaa cca aat gc

gta aat gac gaa aac tac ata ccc atg aat cca gtc aat cct acc gat gct atc
gta aat gac gaa aac tac ata ccc atg aat cca gtc aat cct acc gat gct atc
cat gaa aag gag aag gct gat agc cag aca tgg gaa gat gct ctc cat tcc
cat gaa aag gag aag gct gat agc cag aca tgg gaa gat gct ctc cat tcc
aac ttt ccc gag cac ggc tgc gaa aag ctt gct aag gat ttt gat ctc gac tcc
gat sac cag
gat sac cag
Appendix C – Wing Measurement Data for *chico*<sub>13063</sub> in trans with +, *chico*<sup>1</sup> or Df(2L)flp 147E

Table 9: Average Wing Size Data for *chico*<sup>13063</sup>/+, *chico*<sup>13063</sup>/chico<sup>1</sup>, and *chico*<sup>13063</sup>/Df(2L)flp 147E – females (n=14)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Area (mm²)</th>
<th>Standard Deviation</th>
<th>Height (mm)</th>
<th>Standard Deviation</th>
<th>Width (mm)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>1.52</td>
<td>0.06</td>
<td>1.09</td>
<td>0.05</td>
<td>1.96</td>
<td>0.05</td>
</tr>
<tr>
<td><em>chico</em>&lt;sup&gt;13063&lt;/sup&gt;/+</td>
<td>1.34</td>
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<td>1.05</td>
<td>0.05</td>
<td>1.82</td>
<td>0.07</td>
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<tr>
<td><em>chico</em>&lt;sup&gt;1&lt;/sup&gt;/+</td>
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<td>0.12</td>
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<td>0.07</td>
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<td>0.11</td>
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<tr>
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<td>0.06</td>
<td>1.69</td>
<td>0.09</td>
</tr>
<tr>
<td>Df/+</td>
<td>1.37</td>
<td>0.09</td>
<td>0.98</td>
<td>0.05</td>
<td>1.93</td>
<td>0.08</td>
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<td>1.03</td>
<td>0.06</td>
<td>1.56</td>
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</table>

Table 10: Average Wing Size Data for *chico*<sup>13063</sup>/+, *chico*<sup>13063</sup>/chico<sup>1</sup>, and *chico*<sup>13063</sup>/Df(2L)flp 147E – males (n=14)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Area (mm²)</th>
<th>Standard Deviation</th>
<th>Height (mm)</th>
<th>Standard Deviation</th>
<th>Width (mm)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>1.19</td>
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<td>0.08</td>
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<td><em>chico</em>&lt;sup&gt;13063&lt;/sup&gt;/+</td>
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<td>0.06</td>
<td>1.68</td>
<td>0.09</td>
</tr>
<tr>
<td><em>chico</em>&lt;sup&gt;1&lt;/sup&gt;/+</td>
<td>1.12</td>
<td>0.06</td>
<td>0.94</td>
<td>0.05</td>
<td>1.69</td>
<td>0.05</td>
</tr>
<tr>
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<td>1.00</td>
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<td>1.54</td>
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<tr>
<td>Df/+</td>
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<td>0.06</td>
<td>1.68</td>
<td>0.06</td>
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<tr>
<td><em>chico</em>&lt;sup&gt;13063&lt;/sup&gt;/Df</td>
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<td>0.08</td>
<td>0.90</td>
<td>0.07</td>
<td>1.49</td>
<td>0.06</td>
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Appendix D – Eclosion Time Line Raw Data for \textit{chico}^{13063}/+, \textit{chico}^{13063}/\textit{chico}^1, and \textit{chico}^{13063}/\textit{Df}(2L)\textit{flp} 147E

Figure 27: Eclosion Profile for \textit{chico}^{13063}/+ females

Figure 28: Eclosion Profile for \textit{chico}^{13063}/\textit{chico}^1 females
Figure 29: Eclosion Profile for \textit{chico}^{15063}/\textit{Df} females

Figure 30: Eclosion Profile for \textit{chico}^{15063}/+ males
Figure 31: Eclosion Profile for \textit{chico}^{1s103}/\textit{chico}^1 males

Figure 32: Eclosion Profile for \textit{chico}^{1s103}/\textit{Df}^+ males
Appendix E – *Drosophila* Food Protocol

*Drosophila* “Cordon Bleu” media – FAST recipe

<table>
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<td>CaCl₂</td>
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<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Tegosept</td>
<td>67ml</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>38mg</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>125mg</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>38mg</td>
</tr>
</tbody>
</table>

Cooking instructions:

1. Microwave 1200ml of distilled water on high for 10 min in large glass bowl.

2. Weigh out cornmeal and yeast, add to 800ml of distilled water in a separate beaker, stir on medium level heat on a magnetic stir/hot plate.

3. Weigh out agar, NaKT, CaCl₂, dextrose and sucrose, add to glass bowl, stir and microwave on high for 9 min.

4. Add yeast/cornmeal slurry to glass bowl, mix thoroughly with the hand blender, microwave on high for 4 min, mix with blender.

5. Add 1000ml of distilled water and mix with hand blender. Place bowl inside a zippered pillowcase to cool. Weigh out the antibiotics (alternate between using Amp and Strep).

6. In a 100ml beaker add the Tetracycline to the Tegosept + 16ml of distilled water. Check the temperature of the food – when below 60°C add the Tegosept/Tetracycline mixture. The Amp or Strep can be added directly. Mix well using hand blender.

7. Dispense 35-40ml per bottle or 8-10 ml per vial. Place filled trays into zippered pillowcases until cool, then sprinkle with yeast and plug. Wrap plugged trays in plastic bags and store in 4°C fridge until needed.

8. Tegosept: 100g of methyl-p-hydroxy benzoate in 1 litre 95% EtOH.