

Autonomous Control of Cell and Organ Size by CHICO, a *Drosophila* Homolog of Vertebrate IRS1-4

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Summary

The control of growth is fundamental to the developing metazoan. Here, we show that CHICO, a *Drosophila* homolog of vertebrate IRS1-4, plays an essential role in the control of cell size and growth. Animals mutant for *chico* are less than half the size of wild-type flies, owing to fewer and smaller cells. In mosaic animals, *chico* homozygous cells grow slower than their heterozygous siblings, show an autonomous reduction in cell size, and form organs of reduced size. Although *chico* flies are smaller, they show an almost 2-fold increase in lipid levels. The similarities of the growth defects caused by mutations in *chico* and the *insulin receptor* gene in *Drosophila* and by perturbations of the insulin/IGF1 signaling pathway in vertebrates suggest that this pathway plays a conserved role in the regulation of overall growth by controlling cell size, cell number, and metabolism.

Introduction

During the development of multicellular organisms, growth and patterning are tightly connected. Although significant advances have been made in the understanding of the mechanisms involved in pattern formation, we know comparatively little about how cell and organ growth are regulated in a multicellular organism (Follette and O'Farrell, 1997; Conlon and Raff, 1999; Stern and Emlen, 1999). What are the developmental signals that control body, organ, or cell size, and how are they transduced? Much of our knowledge about growth regulation stems from studies done using tissue culture cell lines. A large number of peptide growth factors have been identified that stimulate cell division and survival of cultured cells. These observations have led to the hypothesis that regulation of overall growth is controlled primarily by coordinating cell cycle progression and cell survival (Conlon and Raff, 1999). Although during development, the regulation of the cell cycle is tightly coupled to morphogenetic events (Edgar et al., 1994), control of overall growth

appears to depend on measuring total tissue volume and not on counting cell number. In mosaic wings consisting of haploid and diploid cells, haploid cells produce a normal sized compartment consisting of twice the number of cells (Santamaria, 1983). Moreover, if mitosis is blocked by use of a temperature-sensitive mutation in *cdc2* (Weigmann et al., 1997) or by overexpression of RBF, a negative regulator of the cell cycle (Neufeld et al., 1998), the result is normal sized compartments consisting of fewer but larger cells. Conversely, accelerating the cell cycle by overexpression of E2F, a positive cell cycle regulator, produces more and smaller cells but does not alter clone size (Neufeld et al., 1998). Thus, changing the length of the cell cycle does not directly affect overall organ growth, which indicates that cellular growth regulation can occur independently of cell cycle control.

In higher vertebrates, hormones and growth factors play an important role in the control of overall growth because they orchestrate cell growth, cell cycle, and cell survival (Conlon and Raff, 1999). Reducing or increasing levels of growth hormone or of its mediators, IGF1 and its receptor (IGFR), dramatically influences body and organ size (for review, Stewart and Rotwein, 1996).

Overall growth (and in some cases cell size) is also affected by the availability of nutrients. Many organisms have developed special survival strategies for periods of growth during low nutrition. Under inadequate nutritional conditions, yeast cells, for example, reduce growth and divide at a smaller size (Thomas and Hall, 1997), whereas nematodes like *C. elegans* enter a diapause called the dauer stage (reviewed in Riddle, 1988). When in this state, larvae arrest development in a sexually immature stage, alter their metabolism to increase the storage of fat, and live up to three times as long as under non-starved conditions. Dauer formation in *C. elegans* is dependent on the cooperation of the insulin and TGF β signaling pathways (Kimura et al., 1997). Raising *Drosophila* under adverse food conditions also results in the production of small flies with fewer and smaller cells (Robertson, 1963; Bryant and Simpson, 1984). Still, little is known in higher organisms about how growth is controlled at the cellular level: what are the genes involved in the regulation of cell growth, and what determines the critical size at which cells undergo mitosis? In *Drosophila*, a class of mutations known as *Minutes* (*M*) dominantly delay development and in some cases reduce body size. Some of the *M* genes encode ribosomal proteins and are thought to slow down growth by reducing protein synthesis. Partial loss-of-function mutations in the *Drosophila myc* gene *diminutive* cause a reduction in overall body size (Gallant et al., 1996). However, it is not yet known how *Drosophila myc* controls growth.

In this paper we present evidence that the *Drosophila* insulin receptor (INR) pathway, and in particular the adaptor protein CHICO, which is homologous to vertebrate insulin receptor substrates (IRS), plays a critical role in the control of cell proliferation, cell size, and overall body growth. We observe that *chico* mutant flies are smaller in size, owing to a reduction in cell size and

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cell number. The effect of *chico* mutations on cell size and cell growth is strictly cell autonomous. In addition to its overall effect on growth, CHICO also controls cellular metabolism; even though *chico* flies are only half the size of normal flies, they show an almost 2-fold increase in lipid levels as compared with their heterozygous siblings. These results provide evidence for a cell-autonomous requirement of the INR signaling pathway in the control of cell size and overall growth.

Results

CHICO Controls Body Size

In a search for mutations causing a reduction in body size, we identified a P element-induced mutation, *fs(2)4¹*, that had been described previously as a female sterile mutation (Berg and Spradling, 1991). Since homozygous *fs(2)4¹* animals are severely reduced in body size, we renamed the mutation *chico*, which means small boy in Spanish. Homozygosity for *chico* causes semilethality and an overall delay in development. Homozygous *chico* flies eclose 2–3 days after their heterozygous siblings. Under noncrowded culture conditions, homozygous *chico* mutant females can produce few viable progeny lacking both maternal and zygotic *chico* function. To quantitate size differences in various mutants, we determined the weight of individual flies (Figure 1B). Flies homozygous for the P element (*chico¹*) or the synthetic *chico* deletion (*chico²*) have a drastic weight reduction (by 65% in females and 55% in males) compared with wild-type control flies of the same age. Body size reduction is observed at all developmental stages but does not alter the overall proportions of the flies (Figure 1A).

Reduced Body Size in *chico* Mutants Is Due to a Reduction in Cell Number and Cell Size

The reduction in body and organ size in *chico* mutants could be due to a reduction in the number of cells and/or a reduction in the size of the individual cells. We determined cell number and cell size in the wing. In the wing, each epithelial cell secretes cuticle with a single hair, so that counting the number of hairs and determining their density provides a direct measure of cell number and cell size in the wing. As shown in Table 1, the 40% reduction in the size of *chico* mutant wings is caused by a reduction in both cell number and cell size. Reduction in cell number accounts for 68% of the total reduction in wing size. The remaining 32% of the reduction in wing size is due to a reduction in the average size of mutant cells. Similar results were obtained for the eye. We have observed that in homozygous mutant *chico* flies, ommatidial number is reduced by approximately 40%: homozygous *chico* flies have only about 480 ommatidia per eye (Table 2), whereas wild-type flies have approximately 780 ommatidia per eye. Therefore, loss of *chico* function reduces body size by means of reducing cell number and cell size.

To test whether the reduction in the size of *chico* mutant cells is also observed during larval stages, we dissociated third instar wing discs of larvae homozygous or heterozygous for *chico* and determined the relative cell size of the two cell populations by FACS analysis (Figure 2). A 10%–14% reduction in the mean of the

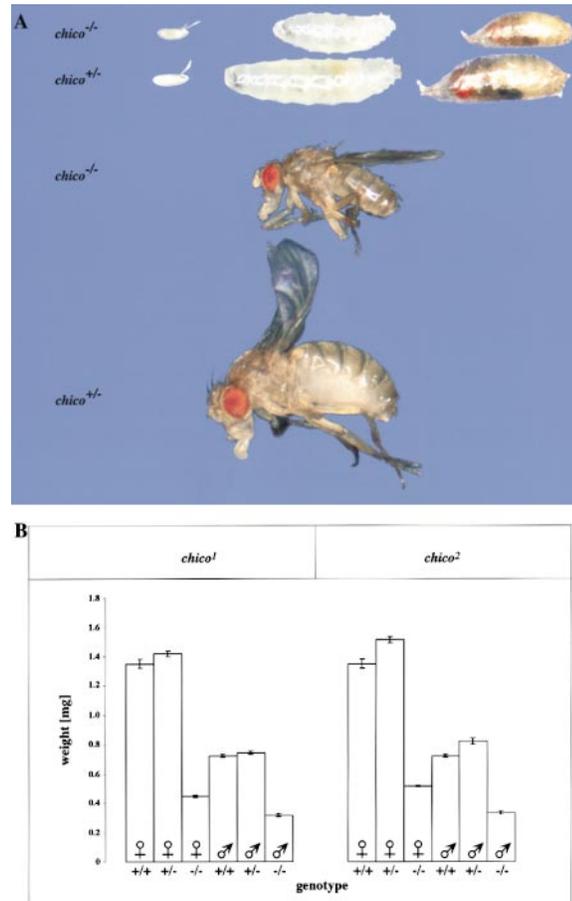


Figure 1. Body Size Reduction of Homozygous Mutant Animals Is Observed at All Developmental Stages

(A) The *chico^{-/-}* egg was derived from heterozygous *chico* females in which *chico^{-/-}* germline clones had been generated. The *chico¹* allele (P element insertion allele) was used for germline clones. In comparison, a heterozygous *chico* mutant egg (*chico^{+/-}*) is shown. Homozygous *chico^{-/-}* larvae, pupae, and adults were derived from heterozygous flies carrying the *chico²* allele.

(B) Body weight of individual flies ($n = 20$) was measured. The weights of *yw* control flies (+/+) and heterozygous (+/-) and homozygous mutant (-/-) *chico* flies are shown.

forward scatter of homozygous *chico* cells compared with heterozygous cells indicates that the size of *chico* imaginal disc cells is also reduced.

chico Encodes a Homolog of Vertebrate IRS1–4

The insertion site of the P element in *chico¹* was mapped 1.5 kb downstream of the *bsk* gene (Figure 3A). Isolation and analysis of partial cDNA clones, a full-length EST clone, and the corresponding genomic sequence flanking the P element insertion indicated that the *chico* gene consists of a single transcription unit of 3.6 kb with nine exons. The P element insertion is located 80 bp downstream of the putative translation initiation site in the PH domain (see below). The open reading frame codes for a protein product of 967 amino acids with a calculated molecular weight of 97 kDa. The CHICO amino acid sequence exhibits the strongest similarity with members of a family of vertebrate insulin receptor

Table 1. Cell Size and Cell Number Are Affected in Wings of Homozygous Mutant Animals

Genotype ^a	1	2	3	4	5	6	7
	Area ^{b,c} (10 ⁶ μm ²)	Overall Size Reduction (%)	Cell Density ^d (Cells/μm ²)	Area Covered per Cell ^e (μm ²)	Cell Size Reduction (%)	Approx. No. of Cells in Measured Area ^{b,f}	Cell Number Reduction (%)
<i>y w; +/Sp</i>	1.47 ± 0.009	—	6.38 × 10 ⁻³ ± 0.1 × 10 ⁻³	157 ± 2.36	—	9379	—
<i>y w; chico²/Sp</i>	1.40 ± 0.018	4.8	6.32 × 10 ⁻³ ± 0.1 × 10 ⁻³	158 ± 2.90	0	8848	5.7
<i>y w; chico²/chico²</i>	0.89 ± 0.009	39.5	7.70 × 10 ⁻³ ± 0.1 × 10 ⁻³	130 ± 1.66	17.2	6853	27

^a From females at least eight wings of each genotype were analyzed.

^b The area of the whole wing was integrated exclusive of the alula and the costal cell.

^c Measured using NIH Image 1.60.

^d Assessed by counting number of wing hairs on the dorsal wing surface in a 10,000 μm² area just posterior to the PCV.

^e Reciprocal of column 3.

^f Generated by multiplying the values in column 1 by those in column 3.

substrate proteins known as IRS1–4. Vertebrate IRS family members are characterized by an N-terminal pleckstrin homology (PH) domain, a phosphotyrosine-binding (PTB) domain, and by a number of phosphotyrosine motifs that can serve as docking sites for SH2-containing proteins (for review, Yenush and White, 1997). Sequence similarity between CHICO and IRS1–4 is confined to the N-terminal region including the PH domain and the PTB domain. The amino acid identity is 41% in the PH domain and 38% in the PTB domain (Figures 3B and 3C). Although there is no significant overall homology within the C-terminal domain, the CHICO protein contains several putative SH2-binding motifs characteristic for IRS family members. Two motifs at positions 411 and 641 fit the consensus binding site (YXXM) for the p85/p60 adaptor subunit of P110 PI(3)K (Songyang et al., 1993; Weinkove et al., 1997), and one (at position 243) corresponds to the consensus (YXN) for GRB2/DRK binding (Olivier et al., 1993; Songyang et al., 1993).

Owing to the insertion of the P element 80 bp downstream of the translation start site, *chico¹* is likely to be a null mutation. The *chico²* allele is a synthetic deletion allele covering the putative translation start site and the regulatory region of *chico* (Figure 3A; see Experimental Procedures). Flies homozygous for this synthetic *chico* null allele were viable and showed phenotypes indistinguishable from flies homozygous for *chico¹* (Figure 1B). The mutant phenotype of both alleles is fully rescued by an 8 kb genomic rescue construct encompassing the *chico* transcription unit and its endogenous regulatory sequences (Figure 3A). Therefore, the reduction in body size and the female sterility are caused by the loss of *chico* function.

CHICO Controls Cell Size Autonomously

The effect of loss of *chico* function on the overall body and organ size could be due to a nonautonomous role of *chico* in humoral growth regulation or to an autonomous role in a tissue- and cell type-specific manner. To test

Table 2. One Mutant Copy of the *Inr* Enhances the Growth Phenotype of *chico* Mutant Cells

Wing analysis ^a							
Genotype	1	2	3	4	5	6	7
	Area ^{b,c} (10 ⁶ μm ²)	Overall Size Reduction (%)	Cell Density ^d (Cells/μm ²)	Area Covered per Cell ^e (μm ²)	Cell Size Reduction (%)	Approx. No. of Cells in Measured Area ^{b,f}	Cell Number Reduction (%)
<i>chico²; +/+</i>	0.96 ± 0.03	—	8.0 × 10 ⁻³ ± 0.12 × 10 ⁻³	125 ± 1.9	—	7,680	—
<i>chico²; Inr⁰⁵⁵⁴⁵/+</i>	0.79 ± 0.03	17.7	7.8 × 10 ⁻³ ± 0.09 × 10 ⁻³	128 ± 1.5	0	6,162	19.8

Eye Analysis^g

Genotype	Eye Area ^c (Arbitrary Units)	No. of Ommatidia per Eye	Area Covered per Ommatidium ^h (Arbitrary Units)
<i>chico²; +/+</i>	261,896 ± 5,097	483 ± 7	542
<i>chico²; Inr⁰⁵⁵⁴⁵/+</i>	198,634 ± 3,280	411 ± 2	483

^a From females four wings of each genotype were analyzed.

^b The area of the whole wing was integrated exclusive of the alula and the costal cell.

^c Measured using NIH Image 1.60.

^d Assessed by counting number of wing hairs on the dorsal wing surface in a 10,000 μm² area just anterior to the PCV.

^e Reciprocal of column 3.

^f Generated by multiplying the values in column 1 by those in column 3.

^g Eight eyes of each genotype were analyzed.

^h Generated by dividing the values in column 1 by those in column 2.

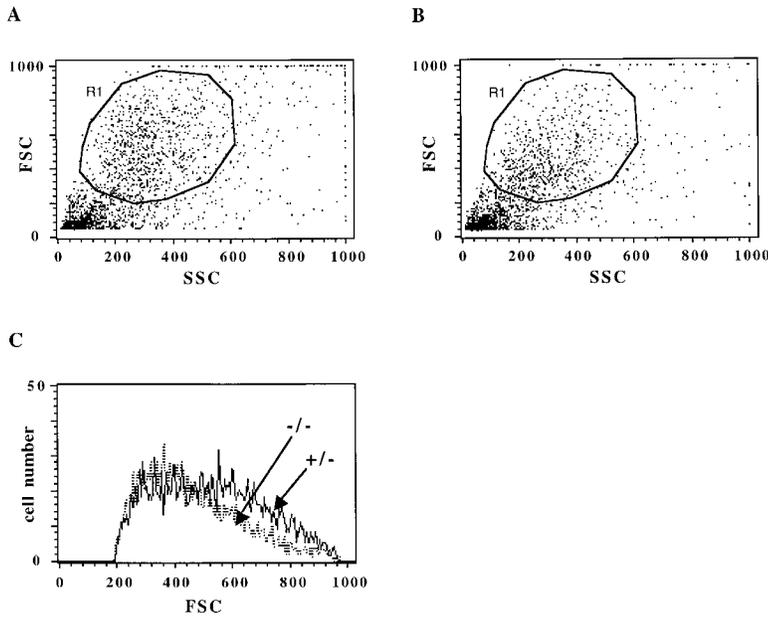


Figure 2. Imaginal Disc Cells of *chico* Mutant Larvae Have a Reduced Size

Comparison of the cell size distribution of heterozygous (+/-) and homozygous mutant (-/-) *chico* wing disc cells determined by FACS analysis. Dot blots of (A) heterozygous (+/-) and (B) homozygous (-/-) *chico* wing disc cells display forward scatter (FSC), which is a measurement for cell size, and side scatter (SSC), which is a measurement for cell granulation. (C) Histogram displays cell size (FSC) and cell number. Comparison of the mean value of the FSC in (A) and (B) of the gated cell populations (R1) revealed that the mean value of the FSC of homozygous mutant *chico* cells is reduced by 10%–14% in three independent experiments compared with the mean value of the FSC of heterozygous cells.

the cell autonomy of the *chico* mutation, we generated clones of genetically marked homozygous mutant *chico* cells in a heterozygous background in the eye (Figure 4A) using the *hs-FLP/FRT* system (Xu and Rubin, 1993). In each ommatidium, the R1–R6 photoreceptor cells are arranged in an asymmetric trapezoid. The tall side of the trapezoid is formed by photoreceptors R1–R3 facing anteriorly. The centrally located R7 photoreceptor has a smaller rhabdomere than the six outer cells. Each of these morphological characteristics is retained in *chico* mutant ommatidia. Thus, loss of *chico* function does not impair the specification of cell fate. However, it is striking that the size of each mutant photoreceptor, and hence the entire ommatidial unit in the mutant clone, is reduced by more than 50%. On the periphery of the clones of homozygous mutant tissue, ommatidia consist of homozygous and heterozygous cells. The genotype of each photoreceptor can be assessed by the presence or absence of red pigment. Small homozygous mutant photoreceptor cells (arrowheads in Figure 4A) coexist with heterozygous cells in the same ommatidium. Remarkably, this does not significantly alter the shape of the ommatidia and the arrangement of the photoreceptors. Autonomy of cell size control is also observed in the wing (data not shown). Therefore, final cell size is autonomously dependent on *chico* function in each individual cell.

CHICO Autonomously Controls Head Size

To test whether *chico* affects the size of organs and body parts autonomously, we selectively removed *chico* function in the eye imaginal disc using the *ey-FLP* technique (B. Dickson, unpublished results). The eye imaginal disc gives rise to the compound eye and the head capsule but not to the proboscis. In embryos heterozygous for *chico*, mitotic recombination was selectively induced in the eye progenitor cells by using an FLP recombinase driven by the *eyeless* enhancer (Quiring et al., 1994). Owing to the presence of a recessive mutation affecting cell survival on the *chico*⁺ chromosome, *chico*

homozygous mutant cells have a proliferative advantage and contribute to the majority of cells in the eye and the head. Thus, flies have heads that are largely homozygous for *chico*, while the rest of the body is heterozygous. In such flies, the eyes and the head capsule are strongly reduced in size, while the proboscis and the rest of the body are of wild-type size (Figures 4B and 4C). Thus, *chico* acts autonomously in the control of cell size and organ size.

chico Mutant Clones Have a Growth Disadvantage but Do Not Show Enhanced Apoptosis

The reduction in cell number caused by the absence of *chico* function may be the result of a prolonged cell cycle time or of an increased rate of apoptosis during development. In order to analyze the behavior of *chico* mutant cells during development, we generated genetically marked homozygous mutant cells by mitotic recombination. This allowed us to compare the behavior of homozygous mutant clones and their wild-type sister clones, called twin spots, generated during mitotic recombination. Three differences between mutant and wild-type twin clones are obvious. First, *chico* mutant clones are rare; in approximately 90% of the clones, only the darkly pigmented wild-type twin spot can be detected. This is most likely due to the fact that small mutant clones encompass only a few ommatidia and escape detection. Second, when a nonpigmented mutant clone is detected, the clone is variable in size and often significantly smaller than the wild-type sister clone. Third, there are regional differences in the frequency of mutant clones: clones are more frequently observed in the anterior half of the eye around the equator. The equator defines a line of dorsoventral mirror image symmetry in the orientation of the ommatidial units. It appears that mutant cells have a better chance to grow or survive in the center of the eye than on its periphery. The behavior of *chico* mutant clones is similar to that of *M* mutant clones and has been described

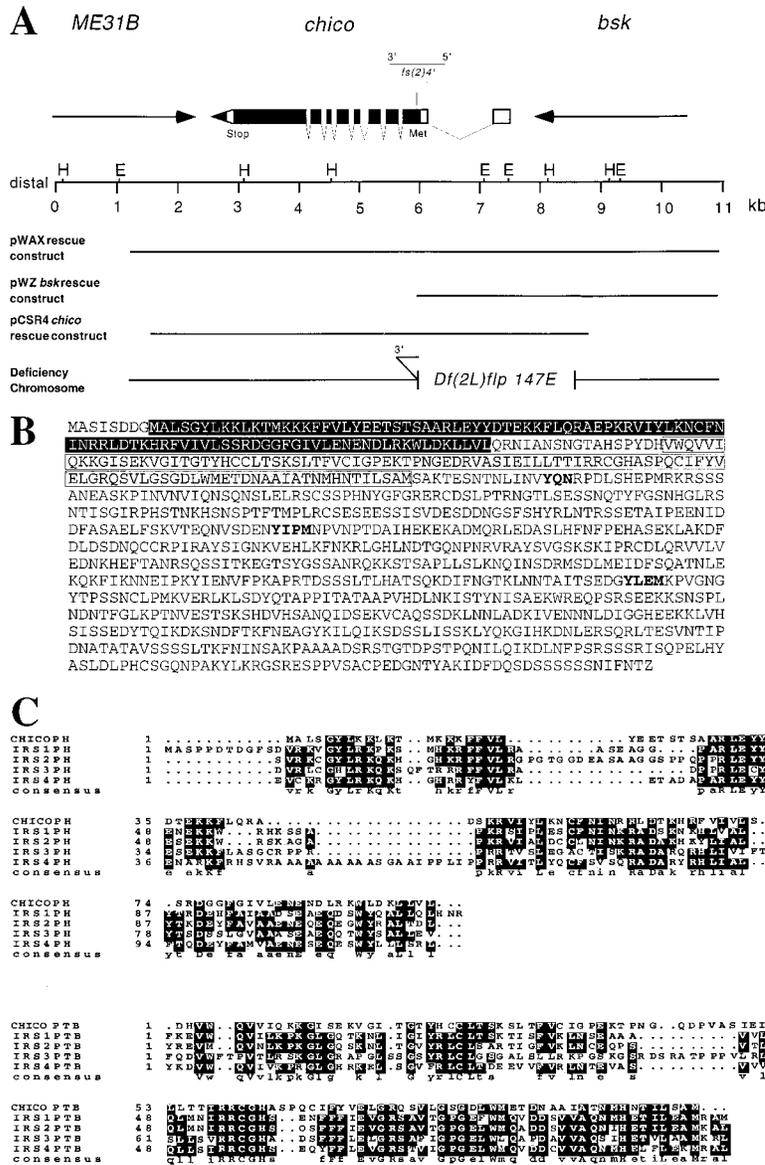


Figure 3. The *chico* Gene Encodes a Homolog of Vertebrate IRS1–4

(A) Genomic structure of the *chico* locus at 31B-C. The putative transcriptional start site lies 221 bp 3' from the end of *bsk* encoding DJNK. *ME31B* encodes a DEAD box RNA helicase (de Valoir et al., 1991). Comparison of the genomic and cDNA sequence revealed that the *chico* transcript contains nine exons with the putative translational start site in the second exon. Black boxes represent translated exons, and open boxes indicate untranslated exons. See text for details concerning the P element insertion, the deficiency chromosome, and the rescue constructs. H, HindIII; E, EcoRI.

(B) Translated amino acid sequence. The black, boxed sequence represents the PH domain, and the gray, boxed sequence represents the PTB domain. Additionally, the putative DRK-binding site (YQN) and the two putative p60 PI3K-binding sites (YIPM and YLEM) are highlighted.

(C) PH and PTB domain alignment. The PH domain and the PTB domain of CHICO were aligned to human IRS-1,2,4 and mouse IRS3. The dark boxes indicate amino acid identity, while the gray boxes indicate amino acid similarity. The lowercase letters in the consensus line represent identity in 3–4 of the proteins, while uppercase letters represent identity in all proteins. The percentage identity was calculated by comparing CHICO individually to each mammalian IRS, and then averaging the four separate identities.

as cell competition (Morata and Ripoll, 1975; Simpson, 1979). It indicates that the development of *chico* mutant cells is selectively impaired compared with wild-type cells and that there are regional differences in the ability of mutant cells to grow or survive.

We went on to test whether the reduced size of *chico* mutant clones observed in the adult is due to a growth disadvantage or to impaired cell survival during the final stages of differentiation by examining mutant clones in the eye and wing imaginal discs. Figure 5 shows eye imaginal discs containing *chico* mutant or wild-type control clones, marked by the absence of the green *arm-lacZ* staining, and their intensely bright green staining twin spots. As seen in the adult, the mutant clones are smaller than their twin spots and are variable in size. The clones often form a thin line. The fact that *chico* mutant clones in the third instar disc and in the adult eye exhibit a similar behavior argues against the possibility that homozygous mutant *chico* cells are eliminated during differentiation in the pupal stage.

Apoptosis has been postulated to be a critical determinant of organ size through counterbalancing cell proliferation (Conlon and Raff, 1999). In order to test whether programmed cell death contributes to size control by reducing cell number, we analyzed discs containing either *chico* mutant clones or wild-type control clones by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). We did not detect a significant difference in occurrence of apoptotic cells between wild-type and mutant clones (data not shown). Since *chico* mutant clones are rather small, we also induced mutant clones in a *Minute* background. Even though such clones were greatly enlarged due to their growth advantage, they also did not reveal enhanced apoptosis compared with wild-type control clones in a *Minute* background (Figures 5C and 5D). Furthermore, we did not observe an increase in morphological signs of programmed cell death, such as enlarged cells or cells with picnotic nuclei in *chico* mutant clones, in the imaginal discs or in the adult eye. These

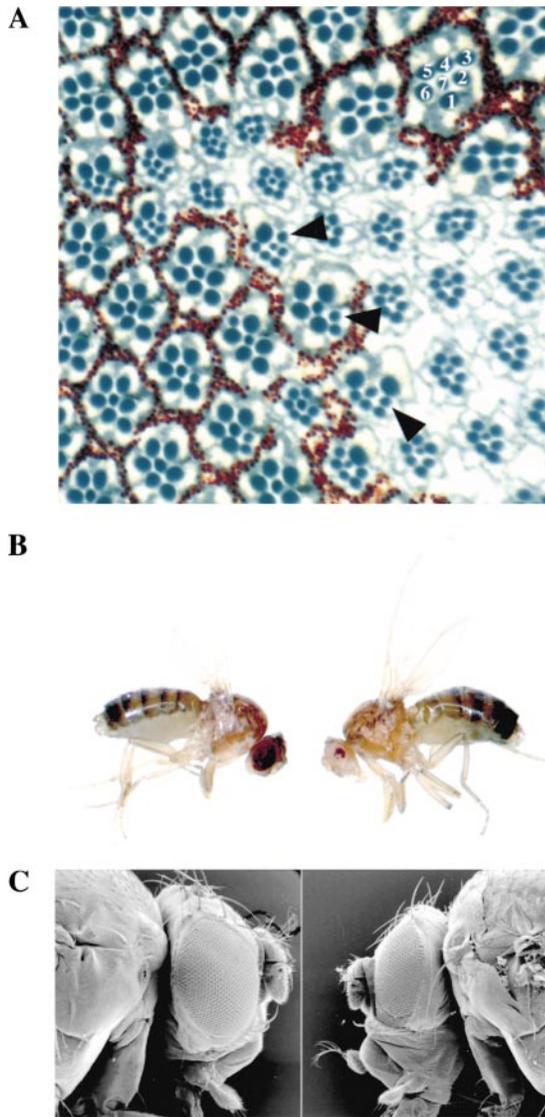


Figure 4. CHICO Controls Cell Size and Organ Size Autonomously
(A) Tangential section through an eye containing a *chico*^{-/-} clone. The *chico*^{-/-} clone is recognized by the lack of pigment. Within the clone, all photoreceptor (PR) cells are reduced in size by about 50% compared with wild-type PR cells (marked by the pigment). At the border of the clone, ommatidia composed of wild-type (marked by the faint yellowish pigment at the base of the rhabdomere) and small *chico*^{-/-} mutant PR cells (arrow head) are visible, indicating that CHICO controls cell size autonomously. The numbers represent PR cells R1–R7.
(B and C) Selective removal of CHICO function in the eye imaginal disc cells generates flies with a strongly reduced head capsule and reduced eyes, whereas the proboscis and the rest of the body are of wild-type size. Flies were of the following genotypes: (B, left panel) *y w ey-Flp; chico*¹ *FRT40/ P(w⁺) I(2)2L-3.1 FRT40; P(w⁺ *chico* genomic rescue construct pCSR4)/+*, (B, right panel) *y w ey-Flp; chico*¹ *FRT40/ P(w⁺) I(2)2L-3.1 FRT40*, (C, scanning electron micrograph, left panel) *y w ey-Flp; chico*¹ *FRT40/ CyO*, and (C, scanning electron micrograph, right panel) *y w ey-Flp; chico*¹ *FRT40/ P(w⁺) I(2)2L-3.1 FRT40*.

results are also consistent with the FACS analysis of heterozygous and homozygous *chico* mutant wing disc cells. We did not detect any significant difference in the apoptotic sub-G1 fraction of homozygous *chico* mutant

cells compared with heterozygous cells (data not shown). Therefore, we conclude that *chico* function is not necessary for cell survival but is required for cell growth and cell proliferation throughout development. Homozygous *chico* mutant cells have a selective growth disadvantage: they grow more slowly than wild-type cells, as indicated by their underrepresentation in discs and in the adult eye, and they cannot reach the normal size of wild-type cells. The cell cycle profiles of heterozygous and homozygous *chico* mutant wing disc cells, however, are similar (data not shown), suggesting that the increased cell cycle time of *chico* mutant cells is caused by proportional expansion of the G1, S, and G2 phase of the cell cycle.

chico Interacts Genetically with the *Drosophila* insulin receptor and PI3 Kinase

The homology of CHICO with mammalian IRS1–4 prompted us to test for genetic interactions with other components involved in signaling via IRS proteins, such as the insulin receptor and the p110 PI3 kinase (PI3K). Loss-of-function mutations in *Inr* are lethal, but certain heteroallelic combinations survive to adulthood. Such *Inr* mutant flies are reduced in size (Chen et al., 1996). We found that, as in *chico* mutants, cell size is reduced by 28% in *Inr*³¹³/*Inr*³²⁷ flies. Furthermore, targeted expression of a dominant-negative variant of *Drosophila* p110 PI3K in the developing eye or wing causes a reduction in cell size in the eye, and in both cell size and cell number in the wing. Conversely, overexpression of a constitutively active, membrane-targeted version of PI3K increases cell size and cell number (Leevers et al., 1996). In flies that are homozygous for *chico*, heterozygosity for a hypomorphic *Inr* allele led to a further reduction in cell number in the wing and the eye (Table 2). Thus, in the absence of *chico* function, a reduction of the receptor level potentiates the growth reduction. This CHICO independent signaling of INR is likely to be mediated by PI3K-binding sites in the C-terminal tail of the INR (Yenush et al., 1996, see Discussion). Similarly, expressing a catalytically inactive version of PI3K in *chico* homozygous wing discs leads to a further reduction in wing size by 48% (data not shown). Thus, the *chico* mutant phenotype is modified by mutations in *Inr* and *PI3K*. This is consistent with the notion that INR, CHICO, and PI3K form a conserved signaling pathway involved in the cell-autonomous control of growth and cell size in *Drosophila*.

CHICO Controls Lipid Levels

Given the role of the insulin signaling pathway in the control of cellular metabolism in vertebrates and in *C. elegans*, we tested whether energy stores are altered in *chico* mutant flies. We determined the amount of lipid, protein, and glycogen per unit of fresh weight. While there was no significant difference in levels of proteins and glycogen, lipid levels were increased significantly in *chico* males (Figure 6). In fact, despite their smaller size, *chico* males had almost twice as much lipids as wild-type males per milligram of fresh weight. The dramatic increase in lipids in *chico* mutant males is reminiscent of hypertriglyceridemia in IRS1-deficient mice (Abe et al., 1998) and of fat accumulation observed in *C. elegans* containing a mutation in the *daf-2* gene, which

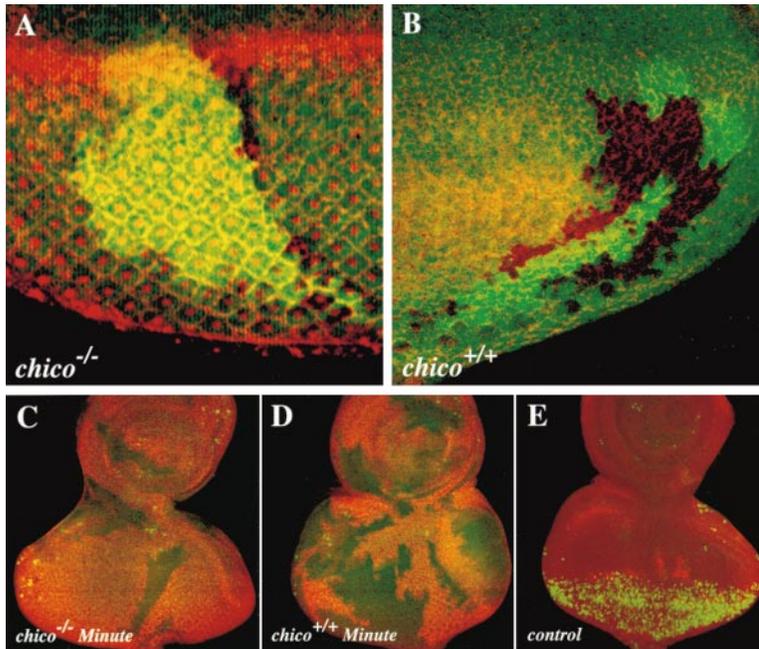


Figure 5. Underrepresentation of *chico* Mutant Cells Is Due to a Selective Growth Disadvantage and Not to Enhanced Apoptosis

Mitotic clones were generated 24–48 hr AED. Clones of *chico* cells are marked by the absence of anti-*lacZ* staining (A and B, non-green; C and D, nonred), their corresponding twin spots by two copies of *lacZ* giving rise to a more intense staining (A and B, light green) and the *chico*-*Minute* double heterozygous tissue by one copy of *lacZ* (C and D, red). Cell membranes are visualized by TR-Phalloidin staining (A and B, red). TUNEL was performed concomitantly for (C)–(E). TUNEL-positive apoptotic cells (C–E, light green) were detected by using a FITC-labeled anti-digoxigenin antibody upon addition of digoxigenin nucleotides to the 3'-OH ends of DNA. (A) A mutant *chico* clone in the eye disc is much smaller than its corresponding wild-type twin spot due to a reduced number of ommatidia.

(B) A *chico* wild-type control clone has about the same size as its corresponding twin spot. (C and D) Eye discs containing either *chico* mutant (C) or *chico* wild-type clones (D) in a *Minute* background. TUNEL-positive apoptotic cells are rare in *chico* mutant (C) and *chico* wild-type clones (D).

(E) As a positive control, eye discs derived from a transgenic line expressing the apoptosis-inducing gene *grim* behind the morphogenetic furrow (MF) under the control of the GMR promoter were coanalyzed. TUNEL-positive apoptotic cells (green) can easily be seen posterior to the MF. Images shown were recorded at identical conditions. Posterior is to the bottom.

encodes the insulin receptor homolog (Kimura et al., 1997). Thus, it appears that the INR signaling pathway controls cellular metabolism in vertebrates, nematodes, and insects.

Discussion

The mechanisms of how body and organ size in a multicellular organism are regulated are largely unknown (Conlon and Raff, 1999). Here, we have provided evidence that loss-of-function mutations in *chico*, which encodes a *Drosophila* homolog of IRS1–4, cause a reduction in overall growth by reducing cell size and cell number. We propose that the insulin receptor signaling pathway in *Drosophila* regulates growth in response to nutritional conditions by the coordinate regulation of cell proliferation, cell growth, and metabolism and that this function has been conserved during evolution.

The Role of CHICO in the Different Aspects of Growth Regulation

Overall growth requires increase in biomass and is normally coupled to cell multiplication. Alterations in cell cycle control can uncouple cell cycle progression and cell size control, but do not alter overall growth (Neufeld et al., 1998). In contrast, loss of *chico* function causes a reduction in growth by reducing both cell number and cell size. Thus, this adaptor protein appears to regulate cell size and cell cycle progression independently. Pattern formation, which is tightly associated with local growth, is unaffected in *chico* mutant flies. Thus, it appears that *chico* controls overall growth without selectively interfering with local growth rates. *chico* function also does not seem to be involved in regulating growth by preventing programmed cell death, since we do not

observe an increase in apoptosis in *chico* mutant clones or in mutant imaginal discs. This is particularly surprising since in mammalian cells the IGF1/PI3K pathway has been proposed to be a survival pathway. It is possible that in developing tissues, such as the *Drosophila* imaginal discs, cells receive survival signals coming from neighboring cells and from the extracellular matrix (Ilic et al., 1998) and do not rely on the same growth and survival factors as isolated cells in culture.

Regulation of growth requires the regulation of protein synthesis. Two kinases, p70S6 kinase and mTOR, regulate protein synthesis in mammalian cells. Each of these kinases is activated by the PI3K/PKB signaling pathway, yet the precise mode of activation is not known (Thomas and Hall, 1997). In *Drosophila*, mutations in the *Drosophila* homolog of *mTor* have not yet been identified. However, loss of *Drosophila* *S6K* activity causes developmental delay and a reduction in body size (J. Montagne et al., submitted). The similarity in the phenotypes observed by loss of *S6K* or *chico* function suggests that CHICO mediates a signal that is at least in part transmitted through the activation of *S6K*. Taken together, these data lead to the conclusion that the primary function of CHICO is to regulate overall growth by coordinating the control of cell cycle progression and cell growth and not by controlling apoptosis.

CHICO May Be Part of a Nutritional Sensing System

Growth is dependent on the availability of nutrients. The reduced body size of *chico* flies is similar to that of flies reared under poor nutritional conditions. Poorly fed flies also eclose later and possess fewer and smaller cells (Robertson, 1963; Bryant and Simpson, 1984). Interestingly, starvation of yeast cells elicits a response very

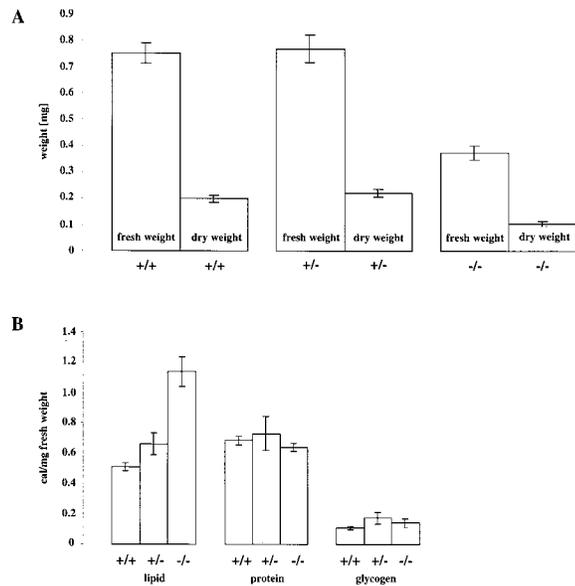


Figure 6. Lipid Levels Are Elevated in Homozygous *chico* Mutant Males

Measurement of different energy stores of individual adult males ($n = 10$) were made. Animals were reared under the same growth conditions and analyzed 3 days after eclosion.

(A) The fresh and dry weight of individual wild-type (+/+), heterozygous (+/-), and homozygous (-/-) *chico* mutant males were determined. The dry weight for wild-type, heterozygous, and homozygous mutant males is approximately 28% of the corresponding fresh weight.

(B) Subsequently, the lipid, protein, and glycogen contents were analyzed. Interestingly, homozygous *chico* mutant (-/-) males contain almost twice as much lipid calories per milligram of fresh weight compared with heterozygous (+/-) or wild-type (+/+) flies.

similar to the one described for *Drosophila*. Under adverse food conditions, yeast cells slow down their cell cycle progression and divide at a smaller critical size (Nurse, 1985). This response may be mediated by the Tor kinase whose regulation of protein synthesis is dependent on the availability of nutrients (Schmidt et al., 1998). While in single-cell organisms nutrient levels directly influence growth, in multicellular organisms a system of overall growth coordination is required, since nutrient conditions are unlikely to be the same for all cells in an organism. Although in *Drosophila* amino acid withdrawal prevents imaginal disc cells and larval neuroblasts to enter the cell cycle, culturing brains of starved larvae in amino acid-rich medium is not sufficient to induce cell cycle entry of quiescent neuroblasts (Britton and Edgar, 1998). When such brains were cocultured with fat body from fed larvae, however, the neuroblasts started to divide. This suggests that growth control is mediated by growth factors secreted from the larval fat body (Britton and Edgar, 1998). Recently, Kawamura et al. (1999) characterized imaginal disc growth factors (IDGFs), which are expressed primarily in yolk cells and fat body. Moreover, IDGFs were shown to cooperate with insulin to stimulate proliferation, and it was speculated that the IDGFs, as chitin-specific lectins, could interact with the INR (Kawamura et al., 1999). Given the fact that in vertebrates, the insulin receptor pathway

plays a critical role in regulating cellular metabolism and growth, it is interesting to speculate that the insulin signaling pathway in flies is part of a nutritional sensing system for each individual cell. Under abundant food conditions, an insulin-like peptide and/or other growth factors, like IDGFs, may be produced and secreted by the larval fat body into the open circulatory system to activate the INR pathway in each cell. As a result, cells grow and divide when a critical cell size has been reached. However, when the level of these growth factors drops because of adverse food conditions, the INR pathway activity is reduced. As a consequence, cells slow down cell cycle progression and cell growth, resulting in fewer and smaller cells.

The increased accumulation of lipids in *chico* adults suggests that in addition to its growth-regulating function, CHICO may also alter cellular metabolism, resulting in the accumulation of lipids. Interestingly, in homozygous *chico* mutant larvae no significant difference in lipid levels was observed. This may suggest that during larval stages *chico* function is required for cell growth and only during pupal development and in the adult CHICO controls metabolism. Thus, at the end of development the INR pathway may control the physiological response required to endure periods of low nutrient conditions.

The Insulin Signaling Pathway Is Conserved in Structure and Function

In mammalian cells, activation of the insulin or IGF1 (insulin-like growth factor 1) receptor by insulin and IGF1, respectively, results in the recruitment of IRS1 or IRS2 to the receptor via interaction of the IRS PTB domains with a phosphotyrosine motif (NPXY) in the juxtamembrane region of the receptors. Phosphorylation of multiple tyrosine residues of IRS1 triggers the activation of various signaling pathways, including the RAS/MAP kinase pathway via the SH2/SH3 adaptor GRB2 and the PI3K/PKB pathway via the p85 SH2 adaptor subunit of p110 PI3K (Yenush and White, 1997). The *Drosophila* INR shares many structural features with its human homologs, including its heterotetrameric structure and a conserved PTB consensus binding site in the juxtamembrane region. However, the *Drosophila* INR contains a 400-amino acid C-terminal extension not found in any of the vertebrate receptors. This C-terminal tail contains three YXXM consensus binding sites for the SH2 domain of the p60 subunit of PI3K and four additional NPXY consensus PTB-binding sites. The C-terminal domain is functional, since expression of a chimeric receptor consisting of the extracellular domain of the human INR and the intracellular domain of the *Drosophila* INR in murine 32D cells lacking endogenous IRS1 can partially activate mammalian PI3K and S6K. In contrast, the ability of the human INR to activate PI3K in this system is strictly dependent on the coexpression of IRS1 (Yenush et al., 1996). These findings and the identification of CHICO suggest that in *Drosophila* INR couples to the downstream effector PI3K in two different ways, one using docking sites in the INR C-terminal tail and the other connecting through docking sites in CHICO.

As described here, the effects on growth and cell size of *chico* mutants are remarkably similar to the phenotypes of mutations in genes encoding other components

of the INR pathway in *Drosophila*. Although loss-of-function mutations in the *Drosophila INR* gene are lethal, certain heteroallelic combinations are viable and show delayed development, reduced body size, and decreased cell number (Chen et al., 1996) and cell size (this study). Expression of dominant-negative or constitutively active variants of p110 PI3K in the developing wing and eye reduces or increases cell number and cell size, respectively (Leevers et al., 1996). Furthermore, viable mutations in the gene encoding *Drosophila* protein kinase B (Staveley et al., 1998) cause a reduction in cell number and cell size (H. S. and E. H., unpublished results). The striking similarities between the phenotypes of *chico* and mutations in the genes encoding INR and DPKB, as well as the genetic interactions between mutations in *Inr*, *chico*, and *PI3K*, emphasize the specific role of the INR pathway in control of cell growth and cell number as a process independent of pattern formation.

The similarities observed in the phenotypes of loss-of-function mutations in the INR pathway in *Drosophila* extend to the phenotypes caused by defects in insulin/IGF signaling pathways in humans and mice. For example, severe insulin resistance in humans causes intrauterine growth retardation and low birth weight (Moller and Flier, 1991). Mice lacking IGF1, IGF1 receptor, IRS1, or IRS2 function are also delayed in development and have a reduced body size (DeChiara et al., 1990; Baker et al., 1993; Liu et al., 1993; Araki et al., 1994; Tamemoto et al., 1994; Withers et al., 1998). Therefore, it appears that the INR/IGFR signaling pathway is conserved from vertebrates to *Drosophila*, not only in regard to its structure but also to its function.

In *Caenorhabditis elegans*, the *Daf-2* insulin receptor homolog and the *Age-1* PI3K homolog also mediate physiological responses to adverse food conditions. In the absence of adequate nutrition or in certain *daf-2* mutants, animals arrest development at the larval stage and change their metabolism to accumulate fat as energy store. These arrested larvae live up to three times longer than worms under normal conditions (Hekimi et al., 1998). Effects of *daf-2* mutants on the control of cell size or cell number have, however, not been observed (G. Ruvkun, personal communication). Furthermore, recent mosaic analysis of the *daf-2* phenotype has shown that DAF-2 controls dauer formation and life span extension in a non-cell autonomous manner (Apfeld and Kenyon, 1998). Therefore, while INR signaling controls a systemic signal involved in diapause and life span regulation in nematodes, growth and cell size are autonomously controlled by the same pathway in *Drosophila*.

Conclusion

The data presented here demonstrate that CHICO controls growth at three different levels: it regulates the size of individual cells, of organs, and of the entire organism. The integrated growth control exerted by CHICO and other components of the insulin signaling pathway makes this pathway a good target for modulating body and organ size during evolution. We have shown that a reduction in insulin signaling by local loss of *chico* function in the developing eye disc reduces head size. Conversely, eye disc-specific overexpression of wild-type PI3K is sufficient to increase eye size (Leevers et al.,

1996). Thus, it is interesting to speculate that genetically controlled modulation of the levels of the insulin receptor or downstream signaling components in different tissues during development has contributed to the different proportions of organs and appendages in closely related species. The high conservation of the insulin signaling pathway suggests that it may have played an important role in the variation of growth control during evolution.

Experimental Procedures

Drosophila Strains

*chico*¹ is a P element insertion allele, originally called *fs(2)4*¹ (Berg and Spradling, 1991). The P element was mapped using standard PCR with primers specific to the 3' end of the P element and to the genomic sequence. Subsequently, the insertion site was precisely determined by sequencing the amplified PCR fragment. *chico*² was derived from *chico*¹ by mobilizing the P element. The resulting *Df(2L)flp147E* deletes the translation start site and the regulatory region of *chico* and the 3' coding sequences of *bsk*. The *bsk* mutation was complemented by insertion of a *bsk* rescue construct on the *Df(2L)flp147E* chromosome (Riesgo-Escovar et al., 1996). For genetic interaction analysis, we used *Dp110*^{D954A}, a dominant-negative form of p110 of *Drosophila* PI3K (Leevers et al., 1996) and *Inr*⁰⁵⁵⁴⁵, a P element-induced hypomorphic allele (Fernandez et al., 1995). The *Dp110*^{D954A} transgene was driven by GAL4, which was expressed in the dorsal wing pouch using the MS-1096 line (Capdevila and Guerrero, 1994). The *Inr* allele is hypomorphic and recessive lethal.

Molecular Characterization of *chico*

An 11 kb genomic DNA fragment that has been described in Riesgo-Escovar et al. (1996) and encompasses the Jun kinase (*bsk*) and *chico* transcription units was used to screen a *Drosophila* cDNA library. From this screen, a partial cDNA (U1) for *chico* was recovered, sequenced, and used to screen an embryonic *Drosophila* cDNA library. Several cDNAs were isolated and partially sequenced (U2–U4). Sequence search of the *Drosophila* EST database with these sequences identified the EST GH02661. Sequencing of this EST clone indicated that it represents a full-length *chico* transcript that contains a consensus sequence for translation initiation (Cavener, 1987) and ends with a poly(A) tail 15 bp after a consensus poly(A) addition signal. All cDNAs were found to encode the same transcript. The 11 kb genomic region was fully sequenced to establish the exon/intron structure of *chico* and also its position in relation to *bsk* and *ME31B*. From a genomic phage of the region, a BamHI/BamHI fragment was subcloned into pBluescript, and a resection from the *bsk* side was performed to generate a 9.5 kb fragment that was subcloned into a transformation vector and used to generate a genomic rescue construct for *chico*. pWAX (described in Riesgo-Escovar et al., 1996) rescued both the phenotypes of *chico* and *bsk* separately and in a double mutant (data not shown; Riesgo-Escovar et al., 1996).

Weight Analysis

Body weight of individual male and female flies (n = 20) was measured with a precision scale (range 0.001–10 mg; Mettler ME30). Flies were reared under the same growth conditions and were age matched (2 days old) before weighing. The genotypes analyzed were the following: *y w; +/+*, *y w; chico*^{2/+} (heterozygotes for the synthetic null allele), *y w; chico*^{2/chico}², *y w; chico*^{1/+} (heterozygotes for the P element insertion allele), *y w; chico*^{1/chico}¹, and *y w; chico*^{1/chico}².

Clonal Analysis

*chico*¹ was recombined onto the FRT40 chromosome (Xu and Rubin, 1993). Germline clones of the *chico*¹ allele were generated using the autosomal dominant female-sterile technique in combination with the Flp recombinase system (Chou and Perrimon, 1996). Females of the genotype *y w; chico*¹, *FRT40/CyO* *y*⁺ were crossed with *y w; hsFlp/Y; P(ovo*^{P1} *w*⁺) *FRT40/CyO* males. Early third instar larvae were heat shocked for 1.5 hr at 38°C. Females of the genotype *y w; hsFlp/y*

w; *chico*¹ *FRT40/P(ovo*^{D1} *w*⁺) *FRT40* were selected and crossed to *chico*¹/*CyO* *y*⁺ males. The resulting progeny lacking any zygotic *chico* function and their siblings bearing the *CyO* *y*⁺ chromosome were analyzed.

For the generation of clones in the adult eye, larvae of the genotype *y w hsFlp*; *chico*¹ *FRT40/ w*⁺ *FRT40* were subjected to a heat shock 24–48 hr AED for 1 hr at 37°C to induce mitotic recombination. Adults were examined for *w* clones and their corresponding twin spots (red pigmented) in the eye. Histological sections of the eyes were done as described previously (Basler and Hafen, 1988). Selective removal of *chico* function in the eye disc progenitors was achieved in animals of the genotype *y w ey-Flp*; *chico*¹ *FRT40/ P(w*⁺) *l(2)2L-3.1 FRT40* and *y w ey-Flp*; *chico*¹ *FRT40/ P(w*⁺) *l(2)2L-3.1 FRT40*; *P(w*⁺ *chico* *genomic rescue construct pCSR4*)/+. The eyFlp technique has been developed by B. Dickson (personal communication).

For the generation of wing clones, larvae of the genotype *f hsFlp/f*; *chico*¹ *FRT40/ck P(f*⁺) *FRT40* were subjected to a heat shock 48–72 hr AED for 0.5 hr at 36°C to induce mitotic recombination. Wings were mounted and examined under a compound microscope.

For the generation of disc clones, larvae of the genotype *y w hsFlp/y w*; *chico*¹ *FRT40/P(arm-lacZ w*⁺) *FRT40* and *y w hsFlp/y w*; *FRT40/P(arm-lacZ w*⁺) *FRT40*, respectively, were subjected to a heat shock 24–48 hr AED for 0.5 hr at 32°C to induce mitotic recombination at a low frequency. Larvae at late third instar stage were dissected. Discs were fixed and permeabilized and stained with appropriate antibodies. Antibodies were rabbit anti-β-Gal (1/2000) and FITC- or TR-conjugated secondary antibodies (1/200). Actin filaments were stained using phalloidin-TR (Molecular Probes).

TUNEL Assay

Apoptotic cells were detected using the ApoptTag system (ONCOR). Clones in larvae of the genotype *y w hsFlp/y w*; *chico*¹ *FRT40/P(arm-lacZ w*⁺) *FRT40* and *y w hsFlp/y w*; *FRT40/P(arm-lacZ w*⁺) *FRT40*, respectively, and *y w hsFlp/y w*; *chico*¹ *FRT40/P(arm-lacZ w*⁺) *M(2L)Z FRT40* and *y w hsFlp/y w*; *FRT40/P(arm-lacZ w*⁺) *M(2L)Z FRT40*, respectively, were induced as described above. Larvae at late third instar stage were dissected. Discs were fixed and stained as described above. 3'-OH ends of DNA were labeled for 0.5 hr at 37°C by addition of digoxigenin 11-UTPs by the enzyme TdT and subsequently detected with FITC-conjugated anti-digoxigenin antibody. Discs of *GMR-grim* larvae, kindly provided by John Abrams (University of Texas), were used as positive controls.

Flow Cytometry

Female larvae of the genotype *chico*²/+ and *chico*²/*chico*², respectively, were dissected at late third instar stage (nonwandering stage). Dissociation of wing discs was done as described in Neufeld et al. (1998). Approximately 20 discs were dissociated. The cell suspension was analyzed using a Becton Dickinson FACStar^{Plus}, and the data were analyzed using Cell Quest (Becton Dickinson).

Metabolic Studies

Adult males (n = 10) of the genotypes *chico*²/*chico*², *chico*²/+ and +/+ were collected 3 days after eclosion. The fresh and dry weight, respectively, from individual males was determined. To determine the dry weight, males were fixed in 100% ethanol for 10 min at 90°C and then dried for 2 hr at 110°C. Protein data were obtained through Kjeldahl digestion and subsequent Nesslerization (Minari and Zilver-smit, 1963), with total nitrogen converted to protein by using a factor of 6.25. Glycogen and lipid data were obtained as described in van Handel and Day (1988).

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