An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control

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Background: Size regulation is fundamental in developing multicellular organisms and occurs through the control of cell number and cell size. Studies in *Drosophila* have identified an evolutionarily conserved signaling pathway that regulates organismal size and that includes the *Drosophila* insulin receptor substrate homolog Chico, the lipid kinase PI(3)K (Dp110), DAkt1/dPKB, and dS6K.

Results: We demonstrate that varying the activity of the *Drosophila* insulin receptor homolog (DInr) during development regulates organ size by changing cell size and cell number in a cell-autonomous manner. An amino acid substitution at the corresponding position in the kinase domain of the human and *Drosophila* insulin receptors causes severe growth retardation. Furthermore, we show that the *Drosophila* genome contains seven insulin-like genes that are expressed in a highly tissue- and stage-specific pattern. Overexpression of one of these insulin-like genes alters growth control in a DInr-dependent manner.

Conclusions: This study shows that the *Drosophila* insulin receptor autonomously controls cell and organ size, and that overexpression of a gene encoding an insulin-like peptide is sufficient to increase body size.

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Background

Each individual organ grows by controlling cell number and/or cell size to reach its final dimensions in relation to the size of the organism. This process is tightly regulated and modulated by environmental factors such as nutrient availability and temperature [1–4]. How organ growth is coordinated within a single individual is still poorly understood. In mammals, hormones and growth factors are known to play a predominant role in controlling organismal growth by orchestrating cell growth, cell proliferation, and cell survival [5, 6]. Reducing the levels of growth hormone or its mediators, IGF1 and the IGF1 receptor (IGF1R), strongly affects body and organ size. In contrast to the well-established role of the IGF1R in growth control, a corresponding role of the insulin receptor is less well understood.

Recently, genetic studies in *Drosophila* have highlighted a conserved signaling pathway that plays an essential role in controlling body, organ, and cell size. This pathway involves the homolog of the insulin receptor substrates (Chico), PI(3)K (Dp110), PTEN (dPTEN), Akt/PKB (DAkt1/dPKB), and S6K (dS6K). Mutations in any one of these components lead to a change in cell size and, with the exception of dS6K, in cell number as well [7–14]. Conversely, overexpression of Dp110 or DAkt1 leads to an increased cell size without affecting cell numbers [9, 13]. Thus, it appears that stimulation of the PI(3)K/PKB pathway alone is not sufficient to promote cell growth and cell cycle progression.

The *Drosophila* homolog of the insulin/IGF1 receptor, DInr, is essential for normal development and is required for the formation of the epidermis and the nervous system during embryogenesis [15]. All described alleles of *dinr* are recessive embryonic or early larval lethal. Only weak heteroallelic combinations of *dinr* alleles were found to be viable and yield adults with a severe developmental delay, small body size, and female sterility [15, 16]. It is not known, however, whether the effect of DInr on growth is cell autonomous and whether activation of DInr is sufficient to promote growth and cell division. Furthermore, the identity of a ligand(s) for DInr has remained elusive.

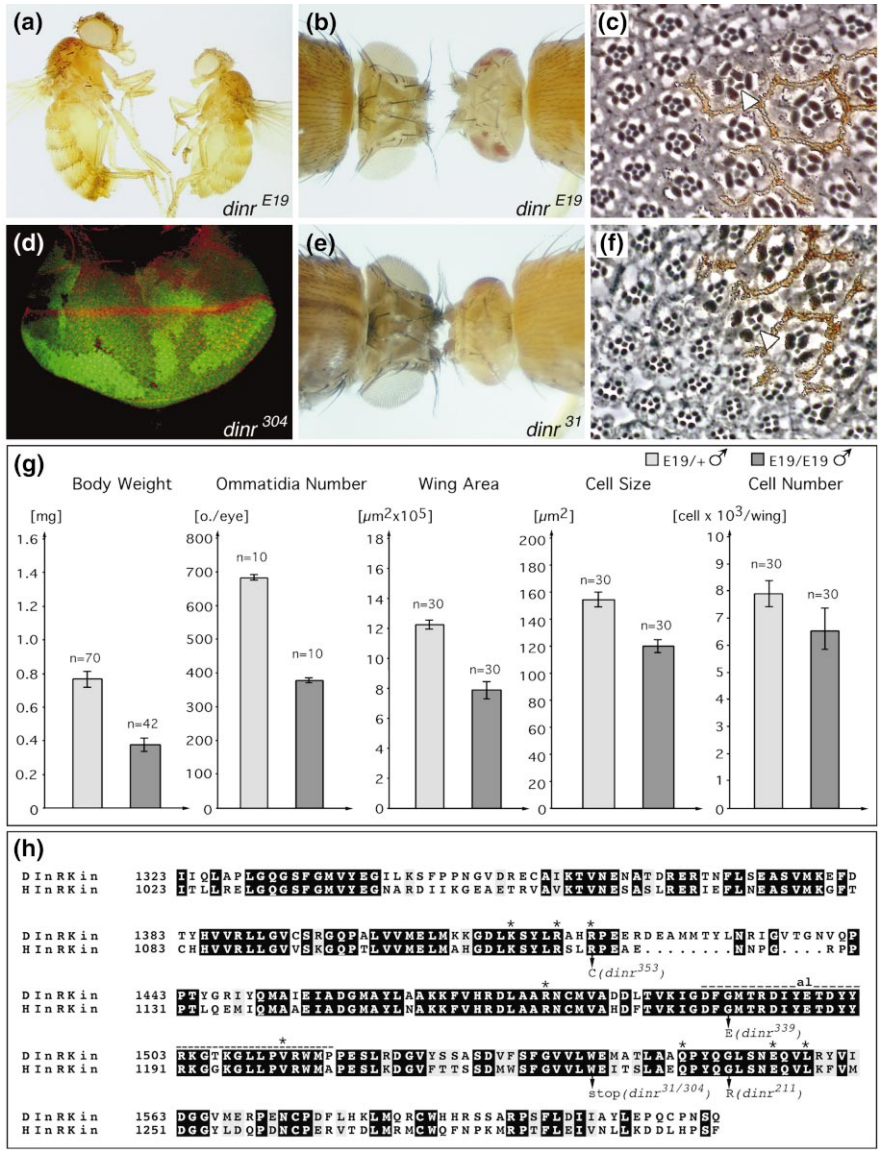
This study examines the effects on cell, organ, and organismal size when activity levels of DInr and of a new putative ligand are changed during development. Furthermore, we describe the structure and expression pattern during development of seven insulin-like genes in *Drosophila*.

Results and discussion

DInr regulates body and organ size by altering cell number and cell size in a cell-autonomous manner

Flies that are homozygous for a partial loss-of-function mutation in *dinr* (*dinr*^{E19}) [16] show a phenotype similar

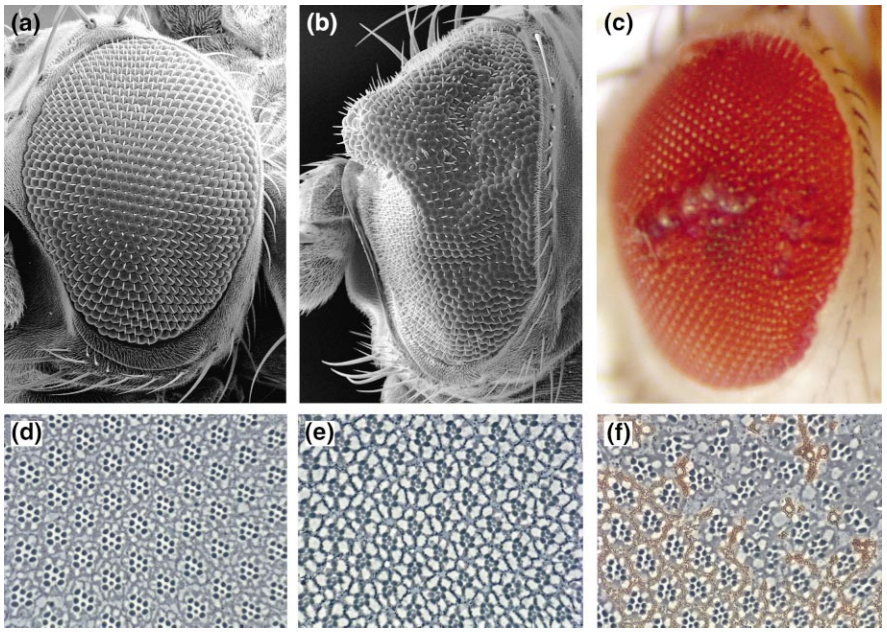
Figure 1



DInr regulates body and organ size by altering cell number and cell size in a cell-autonomous manner. **(a)** *dinr*^{E19}/*dinr*^{E19} flies (right) show a proportionate body size reduction compared to *dinr*^{E19}/+ control flies (left). **(b,e)** Selective removal of DInr function in eye progenitor cells generates flies with strongly reduced eyes and head capsule while all other body parts are of wild-type size. Note the relatively smaller head size of a null allele (e) than of a hypomorphic allele (b) compared to controls. **(c,f)** Tangential sections through a mosaic eye containing small homozygous *dinr* mutant ommatidia (lacking pigment) and normal-sized heterozygous ommatidia (containing pigment). Homozygous *dinr*^{E19} (c) and *dinr*^{31} (f) mutant photoreceptors are reduced in size by approximately one third and more than half, respectively. Arrowheads point to rhabdomeres of small homozygous mutant photoreceptors within genotypically mixed ommatidial units coexisting with normal heterozygous photoreceptors, demonstrating a cell-autonomous requirement for DInr. **(d)** Confocal microscope section of a third instar eye imaginal disc containing mitotic clones of *dinr*^{304} mutant cells (absence of green staining), which are significantly smaller than their wild-type (bright green staining) sister clones; anterior is to the top. **(g)** Quantitation of body and organ size in *dinr*^{E19} homozygous mutant male flies and in heterozygous siblings. In homozygous flies, the body weight and number of ommatidia is reduced by 52% and 45%, respectively. The wing area is decreased by 36%, due to a significant decrease in cell size and cell number by 23% and 17%, respectively. Values are means ± standard deviation; all values of homozygous flies are significantly reduced relative to their heterozygous siblings (t-test, $p < 0.0001$). Similar results were obtained with female flies. **(h)** Five *dinr* alleles carry point mutations in conserved amino acid residues of the kinase domain. An alignment of the kinase domain of the human insulin receptor (HInRKin) and the *Drosophila* homolog of insulin receptor (DInRKin) is shown with the localization and molecular description of the point mutations. *dinr*^{31} and *dinr*^{304} are nonsense mutations in the same codon at different nucleotide positions (Trp1439Stop). The mutations leading to an amino acid substitution are *dinr*^{353} (Arg1419Cys), *dinr*^{339} (Gly1491Glu), and *dinr*^{211} (Gly1551Arg). Asterisks denote predicted active site residues conferring substrate specificity [21]. The shading marks amino acid identity (black) or similarity (gray). We used the amino acid numbering of Fernandez *et al.* [15] for DInRKin and of Ebina *et al.* [45] for HInRKin. The sequences were aligned with the GCG program package. The abbreviations are single amino acid letter code, with "al" indicating an activation loop. Flies were of the following genotypes: (a) *y w; dinr*^{E19}*/TM3, Sb Ser* (left) and *y w; dinr*^{E19}*/dinr*^{E19} (right), (b,e) *y w ey-Flp; FRT82B dinr*^{E19,31}*/TM6B, y*$^{+}$ (left) and *y w ey-Flp; FRT82B dinr*^{E19,31}*/FRT82B P(w*$^{+}$*) 3R3.7* (right), (c,f) *y w ey-Flp; FRT82B dinr*^{E19,31}*/FRT82B P(w*$^{+}$*) 3R3.7*, (d) *y w hsFlp/y w; FRT82B dinr*^{304}*/FRT82B P(arm-lacZ, w*$^{+}$*)*, and (g) *y w; dinr*^{E19}*/+ and y w; dinr*^{E19}*/dinr*^{E19}.

to that previously described for weak heteroallelic combinations. The developmental time is extended from 10 to 20 days, and body size is severely but proportionally reduced. The mutant flies are approximately half the weight of their heterozygous siblings (Figure 1a,g), and females are sterile. Furthermore, like *chico* mutant flies, *dinr*^{E19} flies have an almost 2-fold increase in lipid content (data not shown). The small body size is attributable to a reduction in cell size and cell number by 23% and 17%, respectively (Figure 1g) as revealed by measuring cell density in the wing. Similarly, the average number of ommatidia in the compound eye of mutant male flies is

Figure 2



Overexpression of DInr in the eye leads to a hyperproliferative outgrowth and an increase in cell size. **(a)** Scanning electron micrograph (SEM) and **(d)** tangential section of control eyes. **(b)** SEM of an eye displaying a dramatic outgrowth due to an increase in ommatidia number. This phenotype is caused by the overexpression of *UAS-dinr*^{wt} with *ey-Gal4* in proliferating eye precursor cells. A tangential section of the corresponding eye **(e)** shows that normal ommatidial arrangement and architecture are retained. **(c,f)** Eyes overexpressing *UAS-dinr*^{wt} in clones under the control of the GMR enhancer, which is mainly activated in differentiating cells. Externally, such clones display enlarged ommatidial units compared with the surrounding wild-type ommatidia (c). In tangential sections (f), these clones (less pigment) contain enlarged ommatidia with enlarged photoreceptors and increased interommatidial distance. The magnification in (d–f) is the same. The genotypes are (a,d) *y w; ey-Gal4/+* (b,e) *y w; ey-Gal4/UAS-dinr*^{wt} and (c,f) *y w hsFlp; GMR>w*$^{+}$*; >Gal4/UAS-dinr*^{wt}.

378 ± 8 compared to 683 ± 8 in heterozygous control flies (Figure 1g). We did not observe any dominant size reduction with various *dinr* alleles.

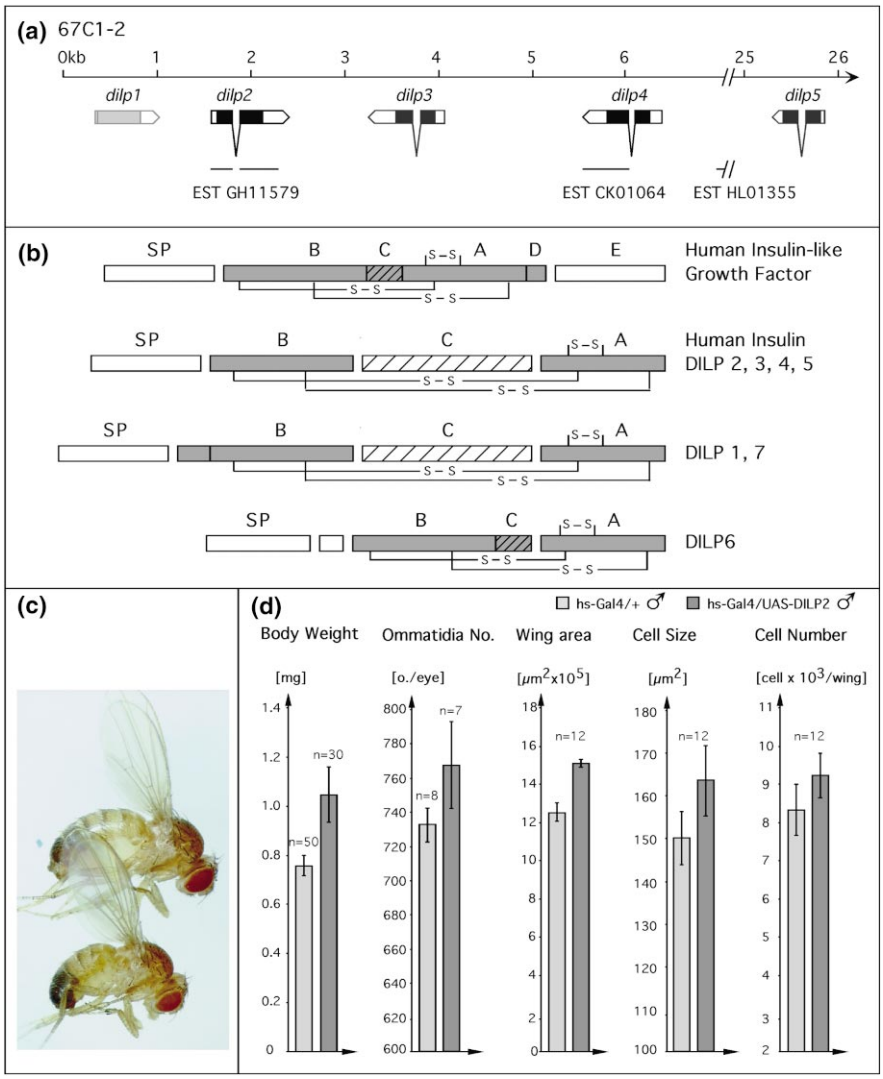
The reduced overall size could be due to DInr acting in the humoral regulation of growth or to it functioning autonomously in a cell- and tissue-specific manner. To test whether DInr affects body parts autonomously, we selectively removed DInr function in the eye imaginal disc using the ey-FLP technique [17]. The eye imaginal disc gives rise to the adult eye and the head capsule. Mosaic flies with heads largely homozygous for various *dinr* alleles displayed a dramatic reduction in eye tissue and in the head capsule, whereas the other body parts were of wild-type size (Figure 1b,e). Notably, the head size was dependent on the allele. This allowed us to arrange the alleles according to their phenotypic strength (compare Figure 1b with 1e). The strongest reduction in head size was observed with *dinr*^{339}, a putative null allele (see below), followed by *dinr*^{31}, *dinr*^{211}, *dinr*^{E19}, and *dinr*^{353}. Thus, DInr regulates head size autonomously.

Comparison of homozygous mutant tissue with heterozygous tissue in tangential sections of mosaic eyes (Figure 1c,f) revealed an estimated reduction in ommatidial size of one third for *dinr*^{E19} homozygous mutant tissue (Figure 1c) and of more than half for a candidate null allele (Figure 1f). Importantly, this growth defect does not impede proper cell fate determination, given that the normal arrangement of the photoreceptor rhabdomeres is retained (Figure 1c,f). Furthermore, the cell size reduction is cell autonomous, as can be seen at the border between homozygous mutant tissue and heterozygous tissue; within the same ommatidial unit, small homozygous cells (arrowhead in Figure 1c,f) coexist with normal-sized heterozygous cells. Although cells lacking DInr function survive and differentiate normally, they have a growth disadvantage compared to heterozygous cells. When homozygous mutant cell clones are induced during early larval life and analyzed in the imaginal discs in the third instar, clone size is greatly reduced compared to the wild-type sister clone (Figure 1d). The phenotypes of *dinr* mutant cells are strikingly similar to those of mutants in the PI(3)K/PKB pathway. Therefore, it is likely that DInr directly regulates cell growth at least in part through the PI(3)K/PKB pathway.

Identification of mutations affecting conserved amino acid residues of the kinase domain of DInr

The structure of DInr is similar to the mammalian insulin receptor (Inr) and the IGF1 receptor (IGF1R) [15]. It is a tetramer composed of two α subunits containing the putative ligand binding domains and two transmembrane β subunits containing the cytoplasmic tyrosine kinase domains. In contrast to human receptors, DInr possesses extensions at the amino and carboxy termini. The C-terminal extension contains binding sites for downstream components similar to those found in insulin receptor substrates (IRS), and has been shown to be able to signal in the absence of IRS proteins [18]. Furthermore, genetic evidence in *Drosophila* suggests that DInr can signal in the absence of Chico, the IRS1-4 homolog [7]. In order to understand the molecular basis for differences in strength of DInr phenotypes, we sequenced the cyto-

Figure 3



A new family of genes encoding putative insulin-related peptides. **(a)** Genomic organization of the *dilp* gene cluster containing *dilp1–5* mapping to 67C1-2 on the third chromosome. Genomic DNA (GenBank accession number AE003550.1) is represented by a line (top) with distances in kb and an arrow pointing to the centromere. Black boxes indicate exons of *dilps* for which mRNA expression has been detected (Table 1). A gray box indicates the predicted exon of *dilp1*, which did not show mRNA expression and therefore may be a pseudogene. Exon–intron boundaries were determined by comparing genomic DNA and expressed sequence tags (EST; bottom lines), or were predicted by the Genscan program [38]. *dilp5* is separated by one intervening gene from *dilp4*. Interestingly, each intron splits the reading frame between position 1 and 2 of a codon; this is typical for insulin-related genes [46]. **(b)** Schematic representation of the predicted structure of *Drosophila* insulin-like peptides (DILP1–7) and comparison with human IGF and insulin. Domains are denoted by letters. The spaces between domains represent predicted proteolytic cleavage [26] during maturation of the propeptide (see also supplementary figure). The active peptides (dark boxes) consist of one polypeptide chain (IGF) or two chains (DILP1–7 and human insulin). Disulfide bonds between conserved cysteines are indicated. **(c)** Proportionate increase in body size of male flies overexpressing DILP2 (top). To achieve ubiquitous expression of DILP2, we used an *hs-Gal4* driver line (Bloomington stock center). Heat shocks at 37°C were applied for 1 hr every 12 hr during development, starting at 24 hr AED. Genotypes are *y w; hs-Gal4/UAS-dilp2* (top) and *y w; hs-Gal4/+* (bottom). **(d)** DILP2 overexpression increases organismal size by increasing the cell size and cell number of individual organs. Compared to control flies, body weight and number of ommatidia are increased by 39% and 5%, respectively. The wing area is increased by 21% due to a significant increase in cell size and cell number by 9% and 11%, respectively. Values are means ± standard deviation; all values of *hs-Gal4/UAS-dilp2* males are significantly increased relative to *hs-Gal4/+* control flies (t-test, $p < 0.001$). Male flies that were 2–3 days old were weighed and analyzed. Similar results were obtained with female flies. Five independent *UAS-dilp2* transgenic lines yielded similar results (n = 16–78).

plasmic region of several *dinr* alleles [15, 16, 19]. In the cytoplasmic portion, 5 out of 22 alleles carry a point mutation. All of them map to conserved amino acid residues within the kinase domain. Two of these point mutations lead to premature stop codons and three are missense mutations (Figure 1h). In humans, most of the mutations that occur within the tyrosine kinase domain of the Inr have been shown to impair insulin-stimulated tyrosine kinase activity [20]. *dinr*^{353} (Arg1419Cys) affects an active site residue, which mediates insulin receptor kinase substrate specificity [21]. Remarkably, a human patient with severe growth retardation associated with insulin resistance, a syndrome called leprechaunism, carries an amino acid exchange at the corresponding position (Arg1092Glu) [22]. It is the only reported homozygous viable mutation in the kinase domain of the human Inr. The patient's parents were heterozygous for this substitution and had severe insulin resistance, but no growth anomalies. Similarly, heterozygosity for *dinr* alleles does not lead to growth phenotypes. These results suggest a role for the insulin receptor in growth control that has been conserved from insects to humans.

Overexpression of DInr in the eye leads to hyperproliferation and an increase in cell size

It has been proposed that a bona fide growth control gene should meet two criteria [6], namely that elimination

Table 1

Summary of *dilp* expression in embryos and larvae.

Gene	Embryo	Larva
dilp1	no signal	N.D.
dilp2	high signal in midgut, low signal in mesoderm stage 12–16	ubiquitous low signal in imaginal discs, high signal in seven cells of each brain hemisphere and in salivary glands
dilp3	no signal	high signal in seven cells of each brain hemisphere
dilp4	high signal in mesoderm stage 2–6, anterior midgut rudiment	high expression in midgut
dilp5	no signal	high signal in seven cells of each brain hemisphere, moderate signal in gut
dilp6	no signal	low signal in gut
dilp7	ubiquitous (except yolk) low signal, moderate signal in midgut	high signal in ten cells of ventral nerve cord

N.D., not determined

should result in growth retardation, whereas overexpression of the gene should promote excessive growth. To determine whether DInr has a direct growth- and proliferation-promoting effect, we overexpressed a wild-type *dinr* cDNA using the UAS/Gal4 system [23]. Expressing *UAS-dinr*^{WT} specifically in proliferating eye precursor cells using an *eyeless-Gal4* driver resulted in a dramatic outgrowth in the adult eye because of an increase in the number of ommatidia (Figure 2b). Histological sections through the overgrown eyes revealed essentially normal cell differentiation but a slight increase in the size of photoreceptor cell bodies (Figure 2e). To further explore the effect on cell size, we overexpressed DInr in clones of cells during cell differentiation. External observation of such clones showed strongly enlarged ommatidia (Figure 2c). Histological sections revealed a cell-autonomous increase in photoreceptor cell size but only a moderate disruption of the ommatidial pattern (Figure 2f). Taken together, these results indicate that DInr activity controls growth in two ways: by regulating cell proliferation and cell size. Interestingly, although overexpression of Dp110 has been shown to increase cell size, it does not increase cell division rates (data not shown) [9]. The IRS homolog Chico contains consensus binding sites for the Drk/Grb2 adaptor and thus may provide a link to the Ras/MAPK pathway. Activation of DInr may promote cell growth and cell division by activation of two signaling pathways. Indeed, MAPK activation is observed in extracts of heads overexpressing an activated form of DInr (Sean Oldham and E.H., unpublished observation).

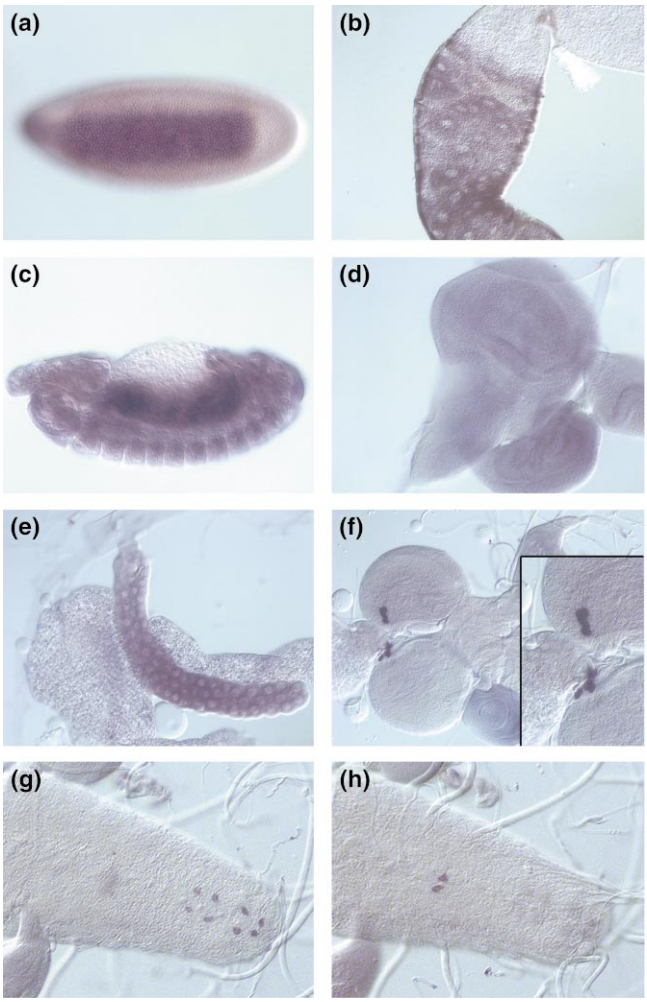
Identification of a new family of genes encoding putative insulin-related peptides

To identify extracellular ligands that regulate DInr activity during development, we searched the *Drosophila* genome [24] for genes encoding insulin-like peptides. Using the conserved spacing of four cysteines within the A chain as a signature for insulin-like peptides [25], we identified seven predicted genes matching these criteria, which we termed *dilp1–7* for *Drosophila i*nsulin-*l*ike *p*eptides. *dilp1–5* are on the third chromosome at cytological position 67C1-2, and constitute a cluster of four contiguous insulin-related genes with *dilp5* separated by one intervening gene from *dilp4* (Figure 3a). The other genes, *dilp6* and *dilp7*, are on the X chromosome at two different loci at cytological positions 2F4 and 3F2, respectively. *dilp1–7* encode putative precursor proteins of 107 to 156 amino acid residues in length that are structurally similar to preproinsulin, with a signal peptide, a B chain, a C peptide, and an A chain (Figure 3b; supplementary figure published with this paper on the internet). Consensus cleavage sites [26] between the B and A chains of all seven DILPs suggest that the active peptides consist of two separate polypeptide chains. Thus, these peptides resemble insulin rather than IGF1 or IGF2, which are single polypeptides (Figure 3b). Comparison of the amino acid sequence of the A and B chains of DILP1–7 with insulin, IGF1, and IGF2 again reveals a higher degree of identical amino acids between these peptides and insulin. DILP2 is the most closely related, with 35% identity to mature insulin (supplementary table). These structural similarities suggest that DILP1–7 are candidate ligands for DInr.

Highly regulated expression of the *Drosophila* insulin genes during development

To determine the expression pattern of the insulin-like genes, we performed in situ hybridization on embryos and larval tissues. The results are summarized in Table 1 and Figure 4. In the embryo, only *dilp2*, *4* (Figure 4a,c), and *7* are expressed at different levels in the mesoderm and midgut. It is interesting to note that the main insulin-producing organs in mammals, the Langerhans islets in the pancreas, are of endodermal origin [27]. Four of the seven genes show a remarkably specific and unique pattern of expression in larvae. *dilp2*, *3*, and *5* display high expression levels in seven cells of anteromedial localization in the brain hemispheres that may correspond to neurosecretory cells (Figure 4f). *dilp3* is exclusively transcribed in these seven cells during larval development,

Figure 4



Spatial and temporal regulation of the expression of *Drosophila* insulin-like peptides. Wild-type embryos (a,c) and third instar larval tissues (b,d–h) after in situ hybridization with *dilp* antisense probes. **(a)** *dilp4* mRNA is detected at the blastoderm stage in the presumptive mesoderm and in the anterior midgut rudiment. Shown is a ventral view. Expression in the mesoderm is still detected after gastrulation, but is reduced from stage 12 onward. **(b)** *dilp4* is reexpressed in the larval midgut. **(c)** *dilp2* shows a broad expression in the embryonic mesoderm starting at stage 12 and an especially intense expression in the midgut, which diminishes at late stage 16. A lateral view is shown. **(d)** *dilp2* is ubiquitously expressed in third larval imaginal discs and in the salivary glands **(e)**. In addition, *dilp2* mRNA is specifically transcribed in seven cells of each brain hemisphere **(f)**. The right panel is a high magnification image. Expression in these seven cells is also detected for *dilp3* and *5*. **(g)** *dilp7* is expressed in a segmental fashion in the ventral nerve cord in four pairs of ventrally located cells in the posterior-most segments and **(h)** in one pair of dorsally located cells in the abdominal segment A1 or A2. Anterior is to the left in (a,c,f,g,h). Note that sense probes of *dilps* did not detect any specific signals in all experiments except for the *dilp2* sense probe, which was uniquely detected in the same seven cells of each brain hemisphere as for the *dilp2*, *3*, and *5* antisense probes. This may be explained by the fact that *dilp3* is located adjacent to and in the opposite orientation of *dilp2* in the genome. Therefore, it is possible that during *dilp3* transcription, the polymerase reads through into the *dilp2* region and, thus, the *dilp2* sense probe could hybridize to the *dilp2* region of the *dilp3* transcript. Consistently, *dilp3* mRNA is also uniquely detected in those seven cells of the brain during development.

whereas *dilp2* (Figure 4c,d,e) and *dilp5* show additional expression domains. *dilp7* mRNA detection is restricted to the ventral nerve cord in a segmental fashion, in four pairs of ventrally located cells in the most posterior abdominal segments and in one pair of dorsally located cells in A1 or A2 (Figure 4g,h). Interestingly, neither of the *dilps* shows detectable levels of expression in the larval fat body.

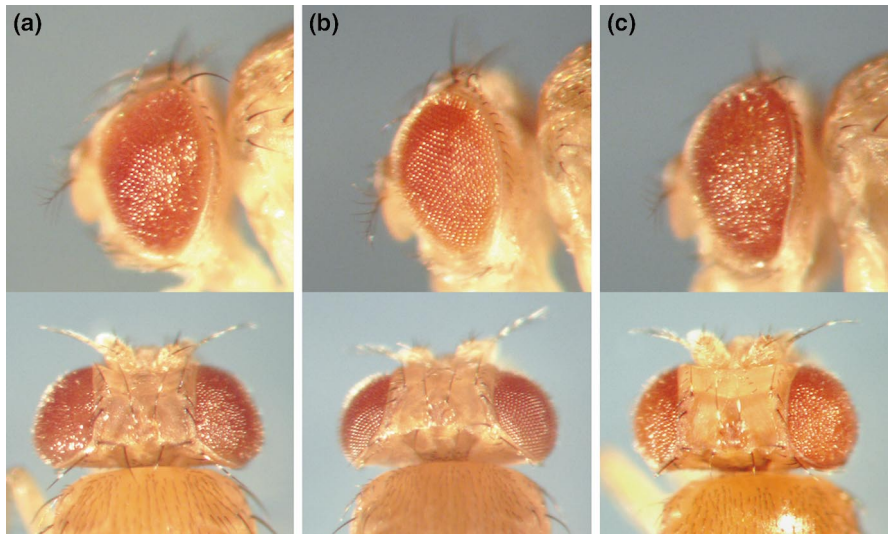
Expression of insulin-related genes in neurosecretory cells has been identified in other invertebrates, such as the insects *Bombyx mori* and *Locusta migratoria* and in the mollusc *Lymnaea stagnalis* [28–30]. In *Bombyx mori*, the neurosecretory cells in the brain are connected to the corpora cardiaca, a secretory gland from which release of insulin-like hormones is triggered by nutrient levels (possibly carbohydrate levels) [31]. We speculate that DILP-expressing neurosecretory cells are connected to the ring gland (the compound endocrine gland of *Drosophila*), which includes the cells of the corpora cardiaca. Release of DILPs from the ring gland may also be under nutritional control. The complex expression pattern of the DILPs, however, suggests a combination of neurosecretory and autocrine/paracrine control mechanisms of cell growth and division during larval development. Mutations in individual *dilp* genes or targeted ablation of specific DILP-expressing cells may help resolve the functions of the *Drosophila* insulins.

DILP2 overexpression increases organismal size by increasing cell size and cell number of individual organs

To gain insight into the function of the DILPs, we overexpressed one insulin-like peptide. For this purpose, we chose DILP2 because it is the closest homolog of human insulin (see supplementary table) and because it is the only DILP with broad expression in imaginal discs (Figure 4d). If DILP2 is a limiting ligand of DInr, we expect that overexpression of DILP2 should promote growth. Indeed, repeated induction of ubiquitous expression of DILP2 during development by means of the UAS/Gal4 system gives rise to bigger flies (39% increase in body weight; Figure 3c,d). Analysis of the eyes of such flies revealed an increase in the number of ommatidia (from 733 $\pm$ 10 to 767 $\pm$ 25 in male flies). Furthermore, quantitative analysis of the wing blade showed an increase in both cell size (by 9%) and cell number (by 11%). These results suggest a role for DILP2 in controlling organismal size by augmenting both cell number and cell size of different organs.

In humans, the in vivo role of insulin as a growth factor is inferred from clinical syndromes, in which excessive

Figure 5



Dominant suppression of a DInr-mediated big eye phenotype by a deficiency uncovering *dilp1–5*. **(a)** Overexpression of *UAS-dinr*^{wt} with the *GMR-Gal4* driver line in differentiating eye cells leads to bulging and rougher eyes. **(b)** *Df(3L)AC1* dominantly suppresses the big eye phenotype caused by the overexpression of DInr. **(c)** Introducing one copy of *UAS-dilp2* efficiently counteracts the suppressive effect of *Df(3L)AC1*. The crosses were performed at 18°C. Flies are of the following genotypes: (a) *y w; GMR-Gal4, UAS-dinr*^{wt}/+, (b) *y w; GMR-Gal4, UAS-dinr*^{wt}/+; *Df(3L)AC1*/+, and (c) *y w; GMR-Gal4, UAS-dinr*^{wt}/*UAS-dilp2; Df(3L)AC1*/+.

insulin secretion results in excessive growth and where a severe deficiency of insulin secretion is associated with poor intrauterine and postnatal growth [32]. For instance, neonates born to women with diabetes in pregnancy or born with Beckwith-Wiedemann syndrome or Nesidioblastosis are macrosomic. In all cases, the growth anomaly is associated with hyperinsulinemia during embryonic development [32–35]. Our demonstration in transgenic flies that overexpression of an insulin-like peptide during development can increase animal size provides further evidence for an evolutionarily conserved role of the insulin pathway in growth control.

DILP2 genetically interacts with DInr

The complementarity between the loss-of-function phenotype of *dinr* and the DILP2 overexpression phenotype (increase in size) suggests that DILP2 may be one of the ligands for DInr. We found that a deficiency (*Df(3L)AC1*) uncovering *dilp1–5* dominantly suppressed the big and rough eye phenotype caused by targeted overexpression of DInr in differentiating eye cells (Figure 5a,b). To test whether the observed dominant suppression was caused by hemizygosity for *dilp2*, we selectively increased the *dilp2* gene dosage by crossing in the *UAS-dilp2* transgene. A single copy of *UAS-dilp2* was sufficient to revert the suppression by *Df(3L)AC1* (Figure 5c), strongly suggesting that *dilp2* is rate limiting for the DInr overexpression phenotype. An analysis of individually mutated *dilp* genes will be required to determine the contribution of the other *dilps* of the cluster (*dilp1* and *3–5*) to the suppressive effect of *Df(3L)AC1*.

To examine whether DInr is limiting for the DILP2 overexpression phenotype, we lowered DInr activity in a DILP2-overexpressing background. Indeed, introducing one mutant copy of *dinr* (*dinr*^{304}) dominantly reduces the increased body weight, cell size, and cell number caused by ubiquitous DILP2 overexpression, indicating a strong genetic interaction between *dinr* and *dilp2* (data not shown). Persistent expression of DILP2 under the control of an *actin* promoter (*Act5C-Gal4*) caused embryonic lethality. This lethality is dependent on normal levels of DInr, as expression of DILP2 in the presence of strongly reduced levels of DInr (see Materials and methods for genotypes) generated viable adults that were small and developmentally delayed. These results are consistent with DInr mediating the effects of DILP2. Furthermore, given that a viable heteroallelic combination of *dPKB* alleles is also able to suppress the embryonic lethal phenotype of DILP2 overexpression, we postulate that the action of DILP2 by DInr is transduced at least in part through the Chico/PI(3)K/dPKB pathway.

Conclusions

In humans, syndromes with mutations in the insulin receptor or with excessive insulin secretion lead to growth abnormalities. This study shows in vivo that altering expression levels of a *Drosophila* insulin-like gene and varying the activity of the *Drosophila* insulin receptor changes the size and number of cells in organs, thereby regulating organismal size. It seems, therefore, that the insulin receptor pathway has been conserved during evolution for a role in growth control from insects to humans. Given the highly tissue-specific expression of the *dilps* in the central nervous system and a broad expression in precursor tissues of adult organs, we propose a nutritionally regulated

mechanism whereby *Drosophila* insulin-like peptides coordinate growth in a neurosecretory and local fashion.

Materials and methods

Generation of mosaic flies by mitotic recombination

The *dinr* alleles (*E19*, *31*, *304*, *211*, *339*, and *353*) were individually recombined onto the FRT 82B chromosome [36]. Mosaic heads were generated with the ey-FLP/FRT recombination system [17], which drives the FLP recombinase under the control of the *eyeless* enhancer, thereby inducing mitotic recombination in eye progenitor cells of embryos that are heterozygous for different *dinr* alleles. A recessive cell lethal mutation on the homologous chromosome prevents the growth of the sister clone. Thus, the *dinr* homozygous mutant clone contributes the majority of cells in the eye and the head capsule. Such flies have heads that are largely homozygous mutant while the rest of the body is heterozygous. To establish an allelic series, we determined the growth defect of each mosaic head ($n = 10$) by measuring the ratio between head and thorax widths and by analyzing the cell size defect in histological sections. The head to thorax ratio of mosaic heads (and control flies) are, in the order of severity, $dinr^{339}$: 0.68 (1.23), $dinr^{31}$: 0.74 (1.20), $dinr^{211}$: 0.79 (1.22), $dinr^{E19}$: 0.81 (1.16), and $dinr^{353}$: 0.82 (1.19). The differences in head size compared to controls and between the alleles analyzed were significant ($p < 0.0001$ and $p < 0.005$).

To generate clones of homozygous *dinr* mutant cells in imaginal discs, *y w hsFLP/y w; FRT82B* $dinr^{304}$*/FRT82B P(arm-lacZ,* w^+*)* larvae were heat shocked 24–48 hr AED for 0.5 hr at 34°C to induce expression of the FLP recombinase. Larvae were dissected at the late third instar stage. Discs were fixed, permeabilized, and stained with mouse anti-β-gal (1/1000) and FITC-conjugated secondary antibodies (1/200).

Measurements of cell number, cell size, and weight

Each cell in the wing blade gives rise to a single wing hair. Thus, the cell density was assessed by counting the number of wing hairs on the dorsal wing surface in a 10,000 μm^2 area just posterior to the posterior cross vein. The reciprocal value of the cell density is the cell area. The approximate number of cells in the whole wing was calculated by multiplying the cell density by the wing area excluding the alula and the costal cell. NIH Image 1.60 was used to measure the wing area. Individual male and female flies were weighed with a precision scale (Mettler ME30, range 0.001–10 mg). Flies homozygous for $dinr^{E19}$ were obtained after removing linked lethal mutations by recombination.

Molecular analysis of dinr alleles

The following *dinr* alleles were sequenced: *E16*, *E19*, *E21*, *31*, *EC34*, *35*, *76*, *87*, *117*, *211*, *242*, *262*, *273*, *277*, *304*, *306*, *310*, *313*, *322*, *327*, *339*, and *353* [15, 16, 19]. Genomic DNA was extracted from heterozygous flies balanced over *TM3 Sb*. The region coding for the cytoplasmic portion was amplified by PCR (primer sequences are available on request), sequenced, and analyzed with Sequencher™ software and compared to the published sequence [15].

Generation of transgenic flies

The *dinr* cDNA was subcloned as an EcoRI fragment into the pUAST vector to generate transgenic flies by means of P element–mediated germline transformation. To achieve expression of *UAS-*$dinr^{wt}$ in proliferating eye precursor cells, the *ey-Gal4* driver line was used [37]. To achieve expression of *UAS-*$dinr^{wt}$ in clones of eye precursor cells undergoing a final round of division and subsequent differentiation, we induced Gal4 expression under the control of the GMR enhancer by heat shocking wandering larvae for 15 min at 34°C containing a heat shock–inducible FLP recombinase and a flp-out transgene (*GMR>*w^+*,STOP>Gal4*). A full-length cDNA clone, EST GH11579 (obtained from Research Genetics), was used to subclone *dilp2* as a 0.65 kb EcoRI/XhoI fragment into pUAST.

Genome search for insulin-like genes, gene structure, and protein structure analysis

We searched for insulin-like genes in the *Drosophila* nucleotide database with the human A chain, which yielded a full-length *dilp2* cDNA clone, GH11579. We then used the predicted *Drosophila* A chains to identify a total of seven insulin-like genes. Searches with A chains were performed with the TBLASTN program (National Center for Biotechnology Information). Gene prediction and conceptual translation was performed with Genscan [38], and signal peptides were predicted using the SignalP program [39]. Cleavage sites were predicted at either specific single or pairs of basic residues of the general formula (R/K)-Xn-(R/K), where cleavage preference decreases with $n = 0$, 2, 4, or 6 [26].

The Berkeley *Drosophila* Genome Project (BDGP) [24] has identified four genes as being insulin-like (corresponding to *dilp1–4*). *dilp5* has not been predicted by BDGP. *dilp5* has its predicted translational start site at nucleotide position 237,598 and stop codon at 237,990 within the contig (nucleotide positions refer to GenBank accession number AE003550.1). We found that the *dilp3* gene consists of two exons and has its translational start at 259,432 and stop at 259,854. Corresponding gene numbers in the genome annotation database of *Drosophila* (GadFly) for *dilp1–4* and *dilp6* and 7 are: CG14173, CG8167, CG14167, CG6736, CG14049, and CG13317.

In situ hybridization

In situ hybridization to larval tissues and embryos was performed essentially as described [40, 41]. The genomic region including *dilp* genes was PCR amplified and cloned into the pCRII-Topo vector (Invitrogen) except for *dilp2* and *dilp4,* which we obtained as an EST clone (LD06542 and GH11579) from Research Genetics. In vitro transcription was done using the DIG RNA labeling kit (Roche). Digoxigenin (DIG)–labeled RNA sense probes were transcribed by SP6 polymerase, and antisense probes by T7 polymerase. Hydrolysis time was shortened to 5 min to prevent cross-hybridization.

Genetic interaction analysis

Df(3L)AC1 (breakpoints 67A2; 67D13, Flybase) removes *dilp1–5*, as *dshc* (67B3) [42], which lies distal to the *dilp* cluster (67C1-2), and *Gap1* (67C2-3) [43], which lies proximal to the *dilp* cluster, are both uncovered by this deficiency. Targeted overexpression of *UAS-*$dinr^{wt}$ in differentiating eye cells by means of *GMR-Gal4* [44] leads to bulging rough eyes (Figure 5a), a phenotype that is dominantly suppressed by *Df(3L)AC1* (Figure 5b). Introducing one copy of *UAS-dilp2* restores the big eye phenotype (Figure 5c). This cannot simply be an additive effect because in a wild-type background, overexpression of *USA-dilp2* under the control of *GMR-Gal4* is phenotypically neutral.

The lethality resulting from overexpression of *UAS-dilp2* with *Act5C-Gal4* (Bloomington stock center) was rescued in the following genotypic backgrounds, in order of decreasing viability: (1) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{05545}$*/*$dinr^{211}$, (2) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{05545}$*/*$dinr^{E19}$, (3) *y w; Act5C-Gal4/UAS-dilp2;* $dPKB^{1}$*/* $dPKB^{3}$, (4) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{E19}$*/*$dinr^{211}$, (5) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{E19}$*/* $dinr^{E19}$, and (6) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{304}$*/+*.

Supplementary material

Supplementary material including a figure showing an alignment of the predicted amino acid sequences of *Drosophila* insulin-like peptides with human preproinsulin, and a table comparing the predicted mature DILP1–7 with human insulin and IGFs are available at http://www.current-biology.com/supmat/supmatin.htm.

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