Insight into Insulin Secretion from Transcriptome and Genetic Analysis of Insulin-producing Cells of Drosophila

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Running title: Drosophila insulin secretion

Keywords: insulin, pancreas, Drosophila, Unc-104, kinesin, Rab1, Golgi, ER, RNA-seq, laser microdissection, transport
Abstract

Insulin-producing cells (IPCs) in the *Drosophila* brain produce and release insulin-like peptides (ILPs) to hemolymph. ILPs are crucial for growth and regulation of metabolic activity in flies, functions analogous to those of mammalian insulin and insulin-like growth factors (IGFs). To identify components functioning in IPCs to control ILP production, we employed genomic and candidate gene approaches. We used laser microdissection and mRNA sequencing to characterize the transcriptome of larval IPCs. IPCs highly express many genes homologous to genes active in insulin-producing beta cells of the mammalian pancreas. The genes in common encode insulin-like peptides and proteins that control insulin metabolism, storage, secretion, and beta cell proliferation, and some not previously linked to insulin production or beta cell function. Among these novelties is *unc-104*, a Kinesin 3 family gene, which is more highly expressed in IPCs compared to most other neurons. Knockdown of *unc-104* in IPCs impaired ILP secretion and reduced peripheral insulin signaling. Unc-104 appears to transport ILPs along axons. As a complementary approach, we tested dominant-negative Rab genes to find Rab proteins required in IPCs for ILP production or secretion. Rab1 was identified as crucial for ILP trafficking in IPCs. Inhibition of Rab1 in IPCs increased circulating sugar levels, delayed development, and lowered weight and body size. Immunofluorescence labeling of Rab1 showed its tight association with ILP2 in the Golgi of IPCs. Unc-104 and Rab1 join other proteins required for ILP transport in IPCs.
Introduction

Signaling through the evolutionarily conserved insulin pathway is critical for organismal homeostasis, controlling everything from growth regulation and development to metabolic homeostasis through glucose and lipid metabolism. In mammals, insulin synthesis and release takes place in pancreatic beta cells. Insulin production is regulated by many factors such as nutrient status and hormonal signals (Newsholme et al. 2010). After translation, insulin is packaged into dense-core vesicles (DCVs) and trafficked to the plasma membrane. Transport of insulin-containing DCVs is microtubule dependent, and the microtubule motor kinesin-1 is known to influence insulin granule transport (Meng et al. 1997; Tabei et al. 2013). DCV transport is additionally regulated by Rab27a. Through its effectors Slac2c, Noc2, Slp4, Exophilin8 and coronin3, Rab27a regulates movement of DCVs and their docking and fusion to the plasma membrane (Yi et al. 2002; Kasai et al. 2005; Kimura et al. 2008; Kimura and Niki 2011; Wang et al. 2013). DCV release is modulated largely via glucose stimulation and internalization, resulting in increased beta cell ATP levels. This induces the closure of ATP-dependent-potassium channels and cell depolarization, triggering an influx of calcium ions through voltage-dependent calcium channels. Ca\(^{2+}\) promotes formation of the SNARE complex allowing DCV fusion and insulin release (Kasai et al. 2010). Thus, proper packaging, trafficking, and exocytosis of insulin-containing DCVs is central to regulating insulin secretion. Defects in insulin production and trafficking arise early in the pathogenesis of diabetes. Many factors involved in DCV trafficking and the molecular details of DCV release remain elusive.

Research in animal models, in particular Drosophila with its large genetic toolkit and fast generation time, can provide mechanistic insights into insulin-like peptide production, and DCV transport and release. Drosophila Insulin-like peptides (ILPs) are homologous to human and mouse insulin/insulin-like growth factors (Brogiolo et al. 2001). Deletion of Ilps 1-5 results in smaller flies with lower metabolic activity (Zhang et al. 2009), while ubiquitous over-expression of Ilp2 is sufficient to promote growth (Ikeya et al. 2002). In flies, ILPs are mainly produced and secreted by insulin-producing cells (IPCs) in the brain to control growth and metabolism (Ikeya et al. 2002; Rulifson et al. 2002). ILP secretion is dependent on autonomous regulation and on inputs received from other cellular populations (Colombani et al. 2003; Geminard et al. 2009; Bai et al. 2012; Rajan and Perrimon 2012). ILPs are also produced by fat body cells during the pupal non-feeding stages (Okamoto et al. 2004).
Flies that lack IPCs have delayed development, reduced growth, and increased circulating sugar levels (Rulifson et al. 2002), suggesting that IPCs in fly play a role comparable to beta cells in mammals.

IPCs number only 14 out of ~100,000 neurons. They develop from a single pair of neuroblasts in the anterior neuroectoderm during late embryogenesis (Wang et al. 2007). During larval stages, IPCs secrete ILPs to promote growth and regulate sugar metabolism, while concurrently undergoing morphological development. Although the morphological development of IPCs during larval stages has not been well characterized, their neuronal processes extend through the brain to the aorta and the corpora cardiac compartment of the ring gland for ILP release (Rulifson et al. 2002). Adult IPCs are important for regulating starvation resistance, responding to oxidative and temperature stress, and adjusting carbohydrate and lipid metabolism (Nassel 2012).

The long neurites of larval and adult IPCs suggests that ILPs require extensive intracellular transport to reach secretion sites, the mechanism of which is largely unexplored. To identify additional cellular components that are important for insulin secretion in vivo, we sequenced mRNA purified from IPCs and identified IPC-enriched gene expression. To obtain a relatively pure population of fly IPCs for mRNA extraction, we used laser-capture microdissection (LCM). Compared with other cell- and tissue-specific RNA isolation techniques for Drosophila, including FACS (Tirouvanziam et al. 2004), magnetic bead-based cell purification (Iyer et al. 2009), and RNA binding protein based strategies (Miller et al. 2009), LCM has advantages for isolating specific cell types, especially for cells that are clustered, like IPCs. LCM has a reasonably high degree of spatial resolution and accuracy (Iyer and Cox 2010). We first characterized the temporal development of IPCs in detail, and analyzed the transcriptome of early third-instar IPCs. We identified 193 genes as enriched in IPCs in comparison to randomly captured neurons, and found that many orthologous genes are active in mammalian pancreatic beta cells. In parallel, we tested 31 YFP-tagged dominant-negative Rab proteins (Zhang et al. 2007) for genes involved in ILP transport. Rab proteins, members of the family of Ras-like GTPases, control many cellular trafficking paths (Stenmark 2009). Our two approaches identified two genes essential in Drosophila for proper trafficking and secretion of ILP-containing DCVs. Here we identify Rab1 as necessary in IPCs to modulate ILP secretion. We discovered a novel function for Unc-104/Kif1a, a
kinesin-3 microtubule motor previously known to transport synaptic vesicles (Okada et al. 1995; Pack-Chung et al. 2007; Barkus et al. 2008), in the transport of DCVs along the axons of IPCs.
Results

IPC morphological changes during development

In preparation for laser dissection, we examined the developmental steps in formation of IPCs to determine optimal conditions and timing for the dissection. Specification of fly brain IPCs during late embryogenesis has been characterized by several studies (Wang et al. 2007; Miguel-Aliaga et al. 2008; Hwang and Rulifson 2011), while post-specification developmental events such as neuronal morphogenesis of IPCs have been little studied. We followed the development of IPC morphology using Ilp2-Gal4-driven membrane (CD8) GFP (Fig. 1 and Supplemental Movies 1-4). At early larval stages, IPCs exist as two symmetrical groups consisting of seven cells in each of the brain hemispheres. Their neuronal processes extend laterally and posteriorly within the brains, with some ending outside the brain, potentially on the aorta and the corpora cardiac compartment of the ring gland (Rulifson et al. 2002) (Fig. 1 and Supplemental Movie 2). At later larval stages, the cell bodies increase in size and their projections extend over greater distances. During pupal stages, the processes that had extended laterally from IPCs during larval stages are gradually dismantled. The processes that initially extended posteriorly from IPCs lengthen and eventually converge into one bundle (Fig. 1 and Supplemental Movie 3). At later pupal stages, the two IPC clusters converge to form one cell group near the midline. During adulthood new processes beneath IPCs are formed. These extend laterally with extensive arborizations. The posterior projection bundle becomes thickened, with extensive arborization at the terminals (Fig. 1 and Supplemental Movie 4). Larval IPCs are necessary for growth control and sugar homeostasis (Rulifson et al. 2002; Haselton and Fridell 2010). The adult IPCs, like larval IPCs, project to the corpora cardiaca and to the aorta for ILP release (Rulifson et al. 2002; Kim and Rulifson 2004; Tatar 2004).

In the third instar, IPCs are clustered in two symmetrically organized groups of seven neurons (Fig. 2A). The neurons’ structural characteristics and arrangement at this stage make them convenient for identification and laser-capture. We chose early third-instar larvae as the IPC source for two reasons: 1) the rapid growth during second and third instars is indicative of the need for IPC-secreted ILPs. 2) Neurite structures of IPCs do not change much during the third instar (Fig. 1), making it more likely that active genes contribute to IPC-regulated ILP production rather than IPC neurite development.
Amplifying sequences of mRNAs extracted from laser-captured larval IPCs

Frozen brain sections containing IPC cell bodies were identified using Ilp2-Gal4>mCD8-GFP and Ilp2-Gal4>nuclearRFP. During larval stages, the Ilp2-Gal4 driver is expressed at low levels in imaginal discs, and at high levels in salivary glands and the 14 brain IPCs. In the brain it is not detectable outside IPCs (Brogiolo et al. 2001; Rulifson et al. 2002). The successful capture of IPCs was demonstrated by the absence of GFP-labeled tissue from the residual sections (Fig. 2B-C). In total, 46 IPC cell bodies from 10 brains were captured and equally divided into two groups (IPC1 and IPC2). Two samples (Control 1, 2) of about 100 non-IPC, non-GFP cells each were collected from the same sections from which IPCs were captured. These adjacent regions of the brain are enriched in neurons that form the superior lateral, superior medial and ventrolateral protocerebrum, dorsolateral neurons, mushroom body, optic tubercle, and lateral horn.

The small amount of RNA isolated from laser-captured cells necessitated amplification of the RNA sequences while trying to minimally skew relative mRNA abundances. We performed two rounds of RNA amplification from each of the four initial samples. In each round of RNA amplification, poly(A)-RNA was reversed transcribed to cDNA, which was then used as template for T7 based in vitro transcription to produce amplified RNA (Wang 2005). One limitation of this RNA amplification method is the lower 5’ representation of amplified RNA due to inefficient reverse transcription at each round of the amplification step (Baugh et al. 2001; Wang 2005) (Fig. 3A). Two rounds of amplification may be insufficient for amplifying the lowest abundance mRNA species. Despite these potential problems, this amplification strategy produces a high correlation of gene expression profiles between unamplified RNA and RNA amplified from low input RNA (500pg of total RNA) (Van Gelder et al. 1990; Wang et al. 2000; Baugh et al. 2001; Lang et al. 2009). To evaluate the fidelity of the LCM and RNA amplification, we used RT-PCR to verify that the major larval Ilp mRNAs were enriched in amplified samples from captured IPCs, not in laser-dissected control samples (Fig. 3B).

After aligning the sequencing reads to the reference D. melanogaster genome and transcriptome (dm3/BGDGP Release 5, from UCSC genome browser, Supplemental Table 1; Materials and Methods) (Adams et al. 2000; Fujita et al. 2011), we deduced the presence of transcripts representing 1851 genes that were common to both IPC samples and 2941 genes that were common to both control samples (The Reads Per Kilobase of total exon length per Million mapped reads (RPKM)≥5.0 for moderate to high
gene expression level, Fig. 3C-D and Supplemental Table 2). The number of expressed genes (total of 3373 from IPC and control samples) is 56% less than the number of genes detectably represented in third-instar larval whole-brain mRNA-seq data (7704 genes, 55% of the total fly genes). The lower transcriptome representation of our laser-dissected samples may be due to: 1. the mRNAs from the 46 captured IPC cells and the ~200 captured cells presumably are from a subset of the genes that are expressed in the whole brain; 2. RNA degradation during LCM and/or insufficient mRNA amplification during IPC and control sample preparation.

Comparing IPCs to the control samples from adjacent regions, 1419 genes are expressed in both. 432 genes are expressed only in IPC samples (IPC1 and IPC2), and 1522 genes are expressed only in control samples (Control 1 and Control 2). Among the 1419 shared genes, the majority showed little variance in gene expression level between IPCs and controls; most of them are clustered around the diagonal (Fig. 3C; see also Supplemental Table 2).

To select mRNAs that are more abundant in IPCs compared to control cells, we used the following criteria: 1) moderate to high expression level in IPCs (RPKM≥5.0 in both IPC samples); 2) higher expression in IPCs than in controls for both replicates (both RPKM_{IPC1} and RPKM_{IPC2} must be greater than RPKM_{Control1} and RPKM_{Control2}); 3) at least two-fold enrichment of expression in IPCs compared to controls (RPKM_{IPC(average)}/RPKM_{Control(average)}≥2.0); and 4) a statistically significant difference between expression in IPCs and expression in controls (p-value≤0.15, by cufflinks/cuffdiff software).

In total, we detected 193 genes that were significantly enriched in IPCs compared to controls (Supplemental Table 3). To identify the biological function groups represented by the IPC-enriched transcripts, we used DAVID, a gene functional classification tool (http://david.abcc.ncifcrf.gov). Genes were grouped into clusters based upon their gene ontology terms (GO terms) to identify genes sharing common biological functions (Table 1 and Supplemental Table 4). Among the IPC-enriched transcripts analyzed by DAVID, the most enriched biological process cluster contains genes encoding the ILPs, G-protein coupled receptors responding to hormone stimulation (Dh31-R, CCHa2-R, and mAChR), and other signaling transducers (Ggamma30A, Ras85D, Hrs, and drl). Genes in this group are essential for ILP production and could be required for IPCs to sense and process upstream neuronal/hormonal signals that act on IPCs. chico and foxo, both functioning in the ILP-receiving cells (Bohni et al. 1999; Puig et
al. 2003), were enriched in IPCs, suggesting a possible autocrine role for ILPs to feed back to IPCs. The second most enriched biological process cluster contains genes encoding sugar metabolic enzymes: \textit{Pfk}, \textit{Mdh2}, \textit{Ald}, \textit{Idh}, \textit{Mdh1}, \textit{CG9467}, and \textit{CG8460}. In mammalian beta cells, glucokinase and the glycolytic intermediates play important roles in glucose sensing (German 1993). Similarly, the enriched sugar metabolic enzymes in IPCs may participate in sugar sensing by IPCs.

Unexpectedly, the third cluster of enriched biological function relates to “skeletal muscle organ development” (Table 1). \textit{Mef2} (Myocyte enhancer factor 2) is the only gene that is annotated as muscle-specific, while most other genes have annotated neuronal and muscular roles, among them neuromuscular junction development. \textit{Mef2} is a transcription factor that functions in both neuron and muscle development in \textit{Drosophila} and is required to control circadian remodeling of clock neurons (Lilly \textit{et al.} 1995; Sivachenko \textit{et al.} 2013). The majority of the genes (\textit{unc-104}, \textit{Frq1}, \textit{Gs2}, \textit{drl}, \textit{Vap-33-1}) in this cluster are involved in synapse assembly and/or synaptic transmission, but have a relatively lower enrichment score due to the presence of \textit{Mef2} in the list. IPCs are neurons, so we believed that “synapse organization” is a more proper representation of genes in Cluster 3. IPCs presumably required such components for organizing IPC axonal terminals and for axonal transport needed for ILP release from these neurons.

Using HomoloGene from NCBI, we searched for mammalian homologs of fly IPC-enriched genes. Of the 193 IPC-enriched genes, 109 have clear mouse homologs (Supplemental Table 5). Transcripts encoding \textit{Drosophila} insulin-like peptides (\textit{Ilp2}, 3, and 5) are the most enriched mRNAs in IPCs (>4000 fold compared with control neural tissue). This confirms that IPCs were included, and enriched, among the captured cells. mRNAs from \textit{amon} and \textit{ia2}, which encode \textit{Drosophila} homologs of mammalian insulin-processing enzymes (Siekhaus and Fuller 1999; Rayburn \textit{et al.} 2009) and insulin-secreting dense-core vesicle component (Harashima \textit{et al.} 2005; Kim \textit{et al.} 2008), were also enriched (8 fold and 11 fold) in IPCs.

**Screening for conserved genes that function in fly IPCs**

Using available \textit{UAS-RNAi} lines, 50 of the 193 IPC-enriched genes were tested for their influence on growth. Transgenes that encode RNAi were engineered to be active specifically in IPCs
RNAi lines for individual genes from either the VDRC or TRiP collections were crossed with *Ilp2-Gal4*, and the adult progeny were examined for their weight (Dietzl et al. 2007; Ni et al. 2008) (Supplemental Fig. 1A). The *Ilp2* promoter drives gene expression in IPCs starting during late embryogenesis and continuing into adulthood (Rulifson et al. 2002; Slaidina et al. 2009) (Fig. 1). RNAi inhibition in IPCs of *amon*, *foxo*, *Rab26*, *unc-104*, *hth*, *ald*, *Pkc98E*, *Vap-33-I*, *Vha26*, and *CG13506* had strong effects on adult fly size (>10% reduction) while inhibition of other genes had little or no effect (Supplemental Fig. 1A).

A recently published mRNA-seq database for genes expressed in adult mouse β-cells was used for cross-species comparison (Ku et al. 2012) (Supplemental Table 5). In this study, the mouse homologs of *amon*, *foxo*, *Pkc98E*, *Rab26*, *Vha26*, *unc-104*, and *Vap-33-I* had high expression levels in pancreatic beta cells compared with other cell types. Amon is the fly homolog of mammalian proprotein convertase subtilisin/kexin type 2 (PCSK2), a processing enzyme for prohormones and neuropeptide precursors. In beta cells, PCSK2 is involved in cleavage of proinsulin to insulin and C-peptide (Davidson et al. 1988). Foxo/Foxo1 has been implicated in beta cell proliferation in the mouse pancreas (Ai et al. 2010). In flies, Foxo functions with JNK to control *Ilp* transcription in response to oxidative stress (Hwangbo et al. 2004; Wang et al. 2005). PRKCE, the mouse homolog of fly *Pkc98E*, is associated with insulin granules for exocytosis upon inositol hexakisphosphate-stimulation (Hoy et al. 2003; Mendez et al. 2003). Mouse Rab37, a homolog of fly *Rab26*, associates with insulin-secretory vesicles based on proteome analysis (Brunner et al. 2007), but its role in insulin secretion is unknown. Unc-104/Kif1a (a kinesin-3 microtubule motor), Vha26 (a proton transport ATPase), and Vap-33-I (vesicle-associated membrane protein) have not been implicated in insulin secretion or beta cell proliferation. We chose Unc-104, a kinesin-3 microtubule motor, for further functional analysis, based upon the strong reduction in growth when it is inhibited in IPCs (Supplemental Fig. 1A).

Unc-104, a kinesin-3 family member, is required for neurite development and transporting ILP along the axons of IPCs

Depletion of *unc-104* mRNA from IPCs during development dramatically reduced adult size (by 27% using a VDRC line and 12% using a TRiP line) (Supplemental Fig. 1A). Unc-104 was previously characterized as an anterograde motor that transports cargos, including neuropeptide-filled and
synaptotagamin-bearing vesicles, in the fly nervous system (Pack-Chung et al. 2007; Barkus et al. 2008). Insulin is packed in DCVs before release (Dean 1973; Takahashi et al. 2004), so in IPCs the ILPs could be transported by Unc-104. To test this possibility, we first determined how ILPs are transported in normal IPCs.

Immunofluorescence labeling of ILP2, one of the major ILPs, together with mCD8-labeled IPC neuronal processes showed that ILP2 is mainly localized in 14 cell bodies and some of their neurites (Fig. 4A). To determine whether these ILP2-positive neurites have axon and/or dendrite features, we expressed axonal (Tau) and dendritic (Khc::nod) markers (Fig. 4B-D) (Rolls 2011). Tau-labeled IPC axonal bundles project contralaterally and posteriorly (Fig. 4B, arrowhead), make a U-turn, and extend anteriorly (white arrow) to terminals outside the brain. ILP2 mainly resides in these axonal projections (Fig. 4A, white arrows; Fig. 4B). The two main populations of Khc::nod-labeled dendritic arborizations extend laterally and posteriorly from the cell bodies (Fig. 4C, yellow arrows). ILP2 was invisible, or at very low levels, in these neurite structures (Fig. 4A, yellow arrows). The pattern of GFP-tagged DCV marker atrial natriuretic factor (ANF) in IPCs showed that DCV-carried neuropeptides are tightly associated with ILP2 granules in IPC cell bodies. This suggests that ILP2, like mammalian insulin, is packaged in DCVs for transport (Fig. 4E) (Dean 1973; Rao et al. 2001; Takahashi et al. 2004). Since Ilp2 mRNA is made in the cell bodies (Brogiolo et al. 2001; Rulifson et al. 2002), ILP2 protein carried in DCVs is transported out of the cell bodies and along the axonal projections of IPCs.

Depletion of Unc-104 from IPCs during early larval developmental stages reduced the number of IPCs, disrupted IPC morphology, and imposed about 1 day of developmental delay in adult eclosion (Fig. 5A), indicating that unc-104 is required during IPC development. Unc-104 is required for fly embryonic motor neuron synapse formation, larval synaptic terminal outgrowth, and dendrite morphogenesis of larval multidendritic neurons (Pack-Chung et al. 2007; Kern et al. 2013). In these past studies, a possible role of Unc-104 in insulin production would have been obscured by early developmental defects. To look specifically at Unc-104 function in ILP production, we employed tub-Gal80ts as a temporal Gal4 switch. Gal80ts inhibits Gal4 until increased temperature inactivates Gal80 and allows Gal4 to trigger gene transcription, in this case transcription of a gene encoding interfering unc-104 mRNA. We allowed Unc-104 to function through late larval development at 18°C, by which time IPC neurite structure has fully developed (Fig. 1), then changed the temperature to 29°C for 24 hrs.
to induce production of *unc-104* RNAi. This strategy successfully prevented occurrence of any visible defects in IPC development (Fig. 5B). In control IPCs with functional Unc-104, low-level ILP2 was detected in the cell bodies. In contrast, 24 hour depletion of *unc-104* mRNA during the second-third larval instar transition caused striking accumulation of ILP2 in IPC cell bodies, as well as enrichment of ILP2 in neurites extending from the IPC cell bodies (Fig. 5B). Quantitation showed that reducing *unc-104* function in IPCs caused a two-fold increase in ILP2 in the cell bodies of IPCs (Fig. 5C). Quantitative PCR analysis indicated no obvious increase in brain *Ilp2* mRNA level after knockdown of *unc-104* for 24 hrs (Supplemental Fig. 2). Thus insulin secretion was likely inhibited after depletion of *unc-104* mRNA.

*Unc-104* is predominantly expressed in the nervous system, in larval and adult flies (Chintapalli et al. 2007; Pack-Chung et al. 2007). To study the role of Unc-104 in IPCs, we examined the localization of Unc-104 protein in IPCs using tagged Unc-104 produced in IPCs (*Ilp2>*Unc-104-*mCherry*, Fig. 5D). Expression of this tagged Unc-104 in the nervous system is sufficient to rescue *unc-104* mutant phenotypes (Pack-Chung et al. 2007; Kern et al. 2013). In IPCs, Unc-104-mCherry strongly colocalized with axons (Fig. 5D). Unc-104-mCherry was also in IPC dendrites, but at a much lower level compared to axons. A similar pattern of Unc-104 localization was observed when GFP-tagged Unc-104 was expressed in IPCs (Fig. 5E) (Barkus et al. 2008).

When *unc-104* was depleted, ILP2 accumulated in IPC axons. The accumulation was limited to regions proximal to the cell bodies (Fig. 5B, bottom panels). In view of the known role of Unc-104 in transporting DCVs in other neurons (Pack-Chung et al. 2007; Barkus et al. 2008), the localization of Unc-104 in IPCs and their axonal processes is consistent with a role for the motor protein in transporting ILP2 in DCVs along axons, especially in regions proximal to the cell bodies.

**IPC-specific production of Rab Dominant-negative proteins identifies Rab1 as a potent growth factor and hemolymph sugar modulator**

To identify additional proteins involved in fly ILP production, 29 dominant-negative fly Rab constructs (*UAS-Rab DN*) were screened by crossing at least one line for each *Rab* gene to the *Ilp2-Gal4* driver (Rulifson et al. 2002; Zhang et al. 2007). Among 43 lines tested, only IPC-specific expression
of Rab1-DN resulted in a dramatic (>40%) reduction in adult fly weight (Fig. 6A; Supplemental Fig. 1B). The other Rab-DN lines, including Rab27-DN, had little effect on fly weights when expressed in IPCs (Supplemental Fig. 1B). Rab27 is the fly ortholog of mammalian Rab27a, which is involved in insulin granule exocytosis (Yi et al. 2002). In the larval brain lobes, Rab27 is expressed in the mushroom bodies and developing antennal lobes, but not in IPCs (Chan et al. 2011), which explains why expression of Rab27-DN in IPCs did not cause a growth phenotype. Expressing Rab26-DN in IPCs had a weaker effect on growth inhibition (7% reduction in size), compared with 20% reduction in size achieved with Rab26 RNAi, suggesting that the DN construct is less effective than the RNAi construct (Supplemental Fig. 1). Rab1 is expressed ubiquitously in the brain including in all 14 IPCs (Fig. 6C) (Chan et al. 2011). Producing Rab1-DN in IPCs dramatically reduced pupal size to only 61% of the controls, in keeping with the RNAi data (Fig. 6B; Supplemental Fig. 3A).

Reducing ILP production or secretion inhibits fly growth and developmental progression (Geminard et al. 2009; Zhang et al. 2009; Gronke et al. 2010). We tested whether inhibiting Rab1 in IPCs affects developmental timing. Flies producing Rab1-DN in IPCs eclosed and pupated, on average, about two days later than control flies (Fig. 6D-E). One direct consequence of reduced ILP production or secretion is elevated levels of circulating sugars in hemolymph, the arthropod circulatory system fluid analogous to mammalian blood (Rulifson et al. 2002). With larvae producing Rab1-DN in IPCs, hemolymph levels of trehalose and glucose were elevated compared to control larvae (Fig. 6F). The average combined trehalose and glucose levels, for control larvae and Ilp2>Rab1-DN larvae, were 2372 mg/dL and 2904 mg/dL respectively. The values of carbohydrate concentration for the Rab1-DN larvae resembled the levels of IPC-ablated larvae (Rulifson et al. 2002). This elevated sugar level, together with developmental delay and growth inhibition caused by Rab1-DN produced in IPCs, suggests that Rab1 promotes ILP production by IPCs.

**Rab1 is required for IPC dendrite morphogenesis and ILP transport out of the IPC cell bodies**

To explore the mechanism by which Rab1-DN inhibits ILP production, we first looked for any IPC developmental defects caused by constitutive inhibition of Rab1 in IPCs. IPC morphology and cell number were examined using mCD8-GFP and a RFP-conjugated nuclear marker, combined with Rab1-DN produced in the IPCs. Under these conditions, there were fewer IPCs in these larvae (Fig. 7A-B).
The average IPC count was reduced from 14 ± 0.0 in wild-type larvae to 8.7 ± 0.4 in Ilp2>Rab1-DN larvae. IPC neuronal morphology was dramatically disrupted. In Ilp2>Rab1-DN larvae, dendritic arborizations were missing (Fig. 7A, white arrows), while the axonal bundles were mostly intact. To specifically examine whether Rab1 controls ILP production, we employed the Gal80ts strategy described earlier to inhibit Rab1 after IPC neurite structures had fully developed. As with unc-104 inhibition, late inhibition of Rab1 for 24 hrs with the tub-Gal80ts system avoided IPC developmental defects but caused a 1.6 fold increase in ILP2 level in IPC cell bodies (Fig. 7C-D). The level of ILP2 was reduced in the axonal projections that connect the IPC cell bodies, suggesting that ILP2 was trapped in cell bodies rather than transported along axons. qPCR assays indicated no obvious changes in brain Ilp2 mRNA level after expression of Rab1-DN for 24 hrs (Supplemental Fig. 2). As an alternative approach to inhibit Rab1 function, we expressed Rab1 RNAi using the Ilp2-Gal4 driver. At 29°C, the RNAi caused strong accumulation of ILP2 in IPC cell bodies but left IPC morphology intact (Supplemental Fig. 3B-D). Brains expressing Rab1 RNAi had the normal 14 IPCs, yet the RNAi reduced fly weight by 20%. The RNAi effect was milder than the effect of Rab1-DN (Supplemental Fig. 3A; Fig. 6A). Rab1 RNAi in IPCs caused only a slight developmental delay (~half day delayed for pupation and adult eclosion), and allowed axons and dendrites of IPCs to be formed and maintained properly. There was some loss of left-right symmetry (Supplemental Fig. 3B), perhaps as a consequence of perturbing global growth. Despite the mild effects on development and IPC morphology, Rab1 RNAi caused a doubling of ILP2 accumulation in IPC cell bodies (Supplemental Fig. 3C-D). Both types of depletion of Rab1 function suggest a role for Rab1 in controlling intracellular trafficking of ILP2.

When Rab1 is inhibited in IPCs, ILP secretion is inhibited during transit from cell bodies to the axonal tracts. To investigate whether Rab1 directly influences ILP transport in IPCs, ILP2 was labeled with antibodies in wild-type IPCs expressing Rab1-YFP. Rab1-YFP was localized within IPC cell bodies with very low fluorescence in IPC axons or dendrites (Fig. 7E). Rab1-YFP and ILP2 immunofluorescent labeling displayed punctate patterns in IPC cell bodies. Strikingly, the majority of ILP2 punctae overlapped with Rab1 punctae, suggesting that Rab1-containing vesicles directly transport ILP granules along the route of ILP secretion.

Rab1 controls ER-to-Golgi transport (Stenmark 2009), so we investigated whether ILP2 granules reside in the Golgi. Producing the GFP-labeled Golgi marker Grasp65 in IPCs revealed punctate Golgi
patterns that co-localized with ILP2 granules (Fig. 7E). As a negative control, the endosome marker FYVE-GFP was produced in IPCs. The FYVE-labeled endosomes were in a punctate pattern, like Grasp65-GFP and Rab1-YFP, but had few overlaps with ILP2 granules. These data, together with the elevated ILP levels in IPCs after Rab1 inhibition, suggested that ILP is delivered by Rab1 to the Golgi in IPC cell bodies. The failed delivery of ILPs outside the cell bodies, due to Rab1 inhibition, results in failed secretion.
Discussion

*Drosophila* brain IPCs regulate development, growth, metabolism, stress resistance, life span, feeding, locomotor activity, olfactory sensitivity, sleep, and ethanol sensitivity in response to internal physiological and external nutritional signals (Nassel 2012). Until now, only a handful of genes were known to function in IPCs. By combining LCM, RNA-seq, two *UAS-RNAi* libraries, and our *UAS-Rab-DN* collection, we systematically screened for genes that are important for IPC function. Among genes that rendered IPC-specific phenotypes when inhibited in IPCs, we focused on *unc-104* and *Rab1* and investigated their roles in ILP secretion and IPC development.

Conservation between mammalian pancreatic beta cells and fly IPCs

Among the neurons, glial cells, intestinal muscle cells, adipocytes, and salivary gland cells that can produce ILPs in flies (Brogiolo et al. 2001; Ikeya et al. 2002; Miguel-Aliaga et al. 2008; Okamoto et al. 2009; Chell and Brand 2010; O’Brien et al. 2011; Sousa-Nunes et al. 2011), brain IPCs are the best-characterized. Several lines of evidence indicate that *Drosophila* brain IPCs and mammalian pancreatic β-cells share functional and physiological similarities: 1) Genetic ablation of fly IPCs or deletion of fly ILPs results in metabolic phenotypes similar to mammals with β-cell/insulin deficiency (Brogiolo et al. 2001; Ikeya et al. 2002; Rulifson et al. 2002; Zhang et al. 2009; Gronke et al. 2010). 2) Fly ILPs and mammalian insulin carry out their functions by activating a conserved signaling pathway in target tissues (Baker and Thummel 2007). 3) In *Drosophila*, brain IPCs and adipokinin hormone-producing cells are physically connected through neuronal projections and in this sense at least are functionally analogous to pancreatic beta and alpha cells (Kim and Rulifson 2004). 4) Beta cells and fly IPCs respond to Leptin/Leptin-like cytokine secreted from adipose tissue to regulate ILP release (Kieffer et al. 1997; Rajan and Perrimon 2012).

Phylogenetic analyses of insulin-producing cells suggest that mammalian beta cells and fly IPCs have common evolutionary origins, or have different origins but have undergone convergent evolution (Arntfield and van der Kooy 2011). Mammalian β-cells arise from the endoderm (Jensen 2004), and fly IPCs are neurons arising from the ectoderm (Wang et al. 2007). Fly IPCs have long axons requiring extensive transport of vesicles, while β-cells have no axons. Yet phylogenic analyses of insulin-expressing cells indicate that neurons evolved to secrete insulin before there were β-cells, creating a
puzzle about the evolutionary origin of β-cells (Arntfield and van der Kooy 2011). In jellyfish, insulin is produced only by neurons (Davidson et al. 1971). In C. elegans and in Drosophila, insulin is produced by neurons and by endoderm-derived cells (Brogiolo et al. 2001; Rulifson et al. 2002; Li et al. 2003). The insulin-producing cells in a worm’s nervous system and intestine respond to food intake to control development, life span, and stress resistance (Li et al. 2003; Iser et al. 2007).

Pancreatic β-cells have some similarities to neurons. From the perspective of physiology: (1) They pack their signaling peptides into secretory granules and release them using action potentials (Rorsman and Renstrom 2003). (2) Hypothalamic neurons can sense blood glucose levels (Lam et al. 2005), like β-cells. From the perspective of gene expression: (1) β-cells express certain “neural-specific” genes, which encode sodium channels, neurofilaments, neurotransmitters (such as GABA), and their receptors (Escurat et al. 1991; Philipson et al. 1993; Glassmeier et al. 1998; Adeghate and Ponery 2002; Xu et al. 2006). (2) Neurons and β-cells do not express the gene encoding neuron-restrictive silencing factor/repressor silencing transcription factor (NRSF/REST), a negative regulator of neuron fate that is made only in non-neuronal cells (Atouf et al. 1997). For all these reasons, β-cells and insulin-expressing neurons may share properties and proteins useful for controlling insulin production and secretion.

By analyzing the IPC transcriptome using LCM and mRNA sequencing, we found a group of IPC-enriched mRNAs that are orthologs of mammalian genes active during insulin production and secretion. Larval-stage specific Ilps (Ilp2, Ilp3, and Ilp5) were found, as expected, plus we detected enrichment of amon, ia2, and Pkc98E mRNAs, which encode Drosophila orthologs of key mammalian insulin-processing enzymes, DCV components, and signal transducers that control insulin secretion (Settle et al. 1995; Siekhaus and Fuller 1999; Hoy et al. 2003; Mendez et al. 2003; Dong et al. 2006; Rayburn et al. 2009). Many other IPC-enriched genes have mouse orthologs that are preferentially transcribed in pancreatic β-cells (Ku et al. 2012). This provided good evidence that our captured samples were highly enriched in IPCs, and strengthened the evidence for conservation between fly IPCs and mammalian beta cells. Functional tests indicated that at least 20% (10 out of 50) of the IPC-enriched genes that have mammalian orthologs are required in IPCs in order for proper Drosophila body size to be attained. RNA interference with expression of any of these genes in IPCs strongly (>10%) reduced growth. Therefore, the combination of LCM and RNA-seq is a powerful and sensitive method.
for characterizing the transcriptome of a specific cell type, such as IPCs, that is present in limited cell numbers in the brain.

Besides ILPs, four neuropeptide genes were enriched in our IPC samples: Myoinhibiting peptide precursor (*Mip*), Dromyosuppressin (*Dms*), neuropeptide F (*npf*), and IFamide (*IFA*). These may be contaminants from adjacent cells (Park et al. 2008). Since our starting materials were only 23 captured cells for each IPC sample, if one of the neuropeptide mRNAs is at a higher level (e.g. >100 fold) compared to average brain cells, it may have been purified along with IPC mRNAs. Since we used third-instar larval IPCs for transcriptome analysis, mRNAs for GPCRs, membrane channels, and transporter proteins that are abundant in IPCs earlier in development may not be present among our enriched mRNAs. Larvae undergo developmental transitions in the third-instar stage that require ecdysone and involve metabolic changes. By capturing gene expression during that time we sampled a larger range of cell properties and conditions than doing the same analysis of adult IPCs.

**IPC neuronal polarity and neurite development**

Our studies and prior work suggest that ILP2 is synthesized in IPC cell bodies and transported out of the cell bodies mainly along Tau-labeled axonal projections to IPC axonal terminals, where it is released (Brogiolo et al. 2001; Ikeya et al. 2002; Rulifson et al. 2002; de Velasco et al. 2007; Geminard et al. 2009). ILP2 is also released within the brain (Bader et al. 2013). By following IPCs during larval development, we observed that IPC axons and dendrites extend over greater distances until the early third-instar larval stage. The neurite extension of IPCs is required to compensate for the size increase of brain lobes, so that connections between IPCs and other brain regions such as subesophageal ganglion are maintained (Rulifson et al. 2002; de Velasco et al. 2007). IPC dendrite extension and more extensive dendritic arborization might also be required to form new neuronal connections with other neurons, thus adjusting to new developmental and physiological needs.

**Unc-104 in ILP transport in IPCs**

Two regulators of ILP production, Unc-104 and Rab1, emerged as the strongest regulators from our screens. Strict regulation of ILP secretion is required to adjust downstream insulin signaling to food availability and metabolic status. Precise control is achieved through regulatory steps in IPCs: sensing
of neuronal or hormonal signals, control of ILP secretion machinery by the signals, directed transport of ILPs to the IPC axonal terminal, and final release of ILP out of IPCs. Based upon our experiments, Unc-104 and Rab1 are both involved in directed transport of ILPs.

Unc-104 in worms and flies, and its mammalian homolog Kif1a, are anterograde motor proteins that transport dense-core vesicles along axons (Okada et al. 1995; Zahn et al. 2004; Pack-Chung et al. 2007; Barkus et al. 2008; Lo et al. 2011). DCVs exist in different cell types. In neurons, DCVs are responsible for transporting, processing, and secreting neuropeptide cargos. In pancreatic β cells, insulin is packed into DCVs. The movement and secretion of insulin-containing DCVs to β cell surfaces is a Ca\(^{2+}\)-dependent process that requires Kinesin heavy chain's movement along microtubules (Meng et al. 1997; Donelan et al. 2002; Cui et al. 2011). It is currently unknown whether other Kinesin family proteins contribute to insulin-containing DCVs movement and secretion. Our study shows that a Kinesin 3 family protein, Unc-104, transports insulin granules along the axons of IPCs. ILP2 colocalizes with DCVs in IPC cell bodies. We observed Unc-104 and ILP2 distributed along IPC axons. Reducing unc-104 function using two different siRNAs caused accumulation of ILP2 in cell bodies and in their proximal axonal projections. Consistent with a role for fly Unc-104 in transporting insulin-like peptides, C. elegans Unc-104 transports fluorescently tagged insulin-like protein 22 and IA-2 in motor neurons (Goodwin et al. 2012). Mammalian Kif1a's role in insulin transport and secretion has not been reported. Kif1a mutant mice die soon after they are born due to severe motor and sensory defects (Yonekawa et al. 1998). They exhibit defects in the localization of synaptic vesicle precursors. Whether newborn Kif1a mutant mice have diabetic symptoms has not been reported.

Rab1 in ILP transport in IPCs

The small GTPase Rab1 regulates membrane trafficking within early Golgi compartments and in the ER-Golgi transition. In Drosophila, Rab1 was first described as a contributor in the maintenance of photoreceptor cell structure by mediating vesicle transport between the rough ER and Golgi body (Satoh et al. 1997). Our study shows that Rab1 is expressed ubiquitously in the fly brain, including IPCs. The specific effect with only Rab1-DN but not other Rab-DNs when expressed in IPCs shows that Rab1-DN interferes specifically with endogenous Rab1 function. It also rules out an alternative interpretation that Rab1-DN titrates some endogenous protein such as a GEF that works on multiple Rabs, one of which is
the real regulator, in addition to Rab1. Inhibiting Rab1 function through dominant-negative or siRNA constructs caused accumulation of ILP2 in cell bodies and substantially reduced ILP2 in IPC axons. YFP-tagged Rab1 is localized predominantly in the cell bodies and exhibits a tight association with ILP2 granules cytologically. A recent study of the silkworm *Bombyx mori* showed that Rab1 is restricted to a small number of neurons in the brain, where it colocalizes with Bombyxin, an insulin family peptide, in the pars intercerebralis area (Uno et al. 2013). Proteomic analysis showed Rab1 is enriched in immunopurified β-cell insulin granules (Hickey et al. 2009). Rab1 may be conserved as a critical molecule needed for insulin production.

**Unc-104 and Rab1 in IPC development**

In addition to their roles in ILP production, Unc-104 and Rab1 are required for IPC development. Constitutive depletion of *unc-104* mRNA from IPCs using *Ilp2-Gal4* resulted in severe disruption of IPC axons and dendrites, and dramatically reduced IPC cell numbers as detected by *Ilp2>mCD8-GFP*. Since IPCs are post-mitotic neurosecretory cells and *Ilp2* mRNA is produced only after IPC differentiation (Wang et al. 2007; Hwang and Rulifson 2011), *Ilp2-Gal4*-driven *unc-104 RNAi* does not interfere with the initial formation and specification of IPCs during late embryogenesis. Therefore, the observed reduction in IPC cell numbers at late larval stages comes from cell death during larval development. This phenotype is consistent with the observation that *Kif1a* mutant mice, and cultures of *Kif1a* mutant neurons, exhibit marked neuronal degeneration and death, which could be caused by insufficient neural stimulation due to disrupted neural connections (Yonekawa et al. 1998). Given the roles that Unc-104 plays in fly motor neuron and multidendritic neuron development (Pack-Chung et al. 2007; Kern et al. 2013), the axonal and dendritic morphology disruption seen in IPCs could be an initial neurite outgrowth defect, a later maintenance defect, or a combination of both.

We have found that constitutive inhibition of *Rab1* in IPCs, using a dominant-negative protein, resulted in disruption of IPC dendrites to a lesser extent than *unc-104 RNAi*. Rab1 functions in ER-to-Golgi transport, so this preferential disruption of IPC dendrites over IPC axons could be explained if IPC dendrites, like those of fly “da” neurons and rodent hippocampal neurons, are more sensitive to the reduction of ER-to-Golgi transport than IPC axons (Ye et al. 2007). *Rab1-DN* induced cell death during IPC development, though less severely than *unc104 RNAi*. The milder phenotypes with respect to
neuron viability and neurite morphology in IPCs producing Rab1-DN compared to IPCs expressing unc-104 RNAi are consistent; a milder defect in neurite morphology may underlie a milder defect in neuron viability. Alternatively, Rab1-DN might cause accumulation of cytotoxic components such as misfolded alpha-synucleins in IPCs, which could underlie the loss of IPCs (Cooper et al. 2006).

Proteins like Rab1 and Unc-104 have multiple functions in a variety of cell types. Using cell-type specific interference, we have explored their roles in production and transport of insulin-like peptides and determined the cellular and whole organism phenotypes associated with their damaged functions. They join an important list of critical factors needed for the controlled production and release of insulin-family proteins.
Materials and Methods

Fly strains and genetics

Fly lines used were listed in Supplemental Table 6.

Temperature shift for Gal80ts experiments:

Embryos were collected on a molasses cap within a 4-hr period at 25°C followed by 40-hr incubation at 18°C. 30 newly hatched first instar larvae were then transferred to each vial, kept at 18°C for another 4 days, and shifted to 29°C for 24 hrs before dissection.

2x fly food (3.4% yeast, 8.3% cornmeal, 6% sucrose, 1% agar) was used to raise flies (Geminard et al. 2009).

Laser microdissection

The method was adapted from Spletter et al (Spletter et al. 2007). Early 3rd-instar larval brains were dissected and quickly aligned and frozen with optimal cutting temperature compound (Tissue Tek; OCT). We sectioned from the posterior end of the mouth hook to the posterior end of the brain lobes, the position of which was marked by the anterior end of a pupa at one end of the block. A dehydration series (distilled H2O for 1min, 50% ethanol for 30s, 70% ethanol for 30s, 95% ethanol for 30s, 100% ethanol for 30s, 100% ethanol for 2 mins, xylene for 2 mins, xylene for 5 mins) was followed before each section (10µm thick) was examined under fluorescence with a 20x objective to identify sections that contain IPCs. IPCs were marked by expression of a genetically encoded GFP reporter (an Ilp2-Gal4 driving a UAS-mCD8-GFP reporter gene). In addition, UAS-nuclearRFP was included in the same fly line to independently label IPC cell bodies with RFP. This double labeling of IPCs, together with strong expression of mCD8-GFP (2 copies) in these neurons, significantly sped up the process and reliability of IPC identification in cryosections. IPC-enriched samples were captured into PCR tube caps on a Leica LCM microscope (model ASLMD) using Laser Microdissection software version 4.4.

RNA isolation, amplification, and mRNA sequencing library construction

Total RNA of laser-captured samples was extracted using Arcturus PicoPure RNA Isolation Kit (Molecular Devices). mRNA was amplified in two rounds using Arcturus RiboAmp HS PLUS
Amplication Kit (Molecular Devices). Amplified mRNA from each sample was fragmented to 100-200nt using 10X RNA fragmentation buffer (Ambion). Fragmented RNAs were ligated to the 3’ linker [“Linker-1”, IDT Inc.] and 5’ linker [5’-ACGCTCTTTCCGATCTv-3’ (uppercase, DNA; v=barcodes with four RNA molecules: cugg, cguc, acuu, or cccu; IDT Inc.)]. cDNA was amplified with 18 PCR cycles, using forward primer 5’-GATACGGCGACCTACGATCCTTCTCCCTAACGTACATT-3’ and reverse primer 5’-CAAGCCAGTAGATGCTTCCCACT-3’, to produce sequencing libraries for the Illumina GA II sequencing system. The details of the RNA library construction protocol are available in the Supplemental Materials and Methods section.

For whole brain (Elav-Gal4> w1118), total RNA from third instar larval brains was extracted using Trizol (Invitrogen) following manufactural instructions. No mRNA amplification was used. mRNA sequencing libraries were constructed using TruSeq RNA sample preparation kit (Illumina).

**Mapping sequencing reads and mRNA profiling**

Barcode splitting was performed on the FASTQ file. The first four bases of each read were compared to the barcodes and up to one mismatch was allowed in the barcode bases. After barcode splitting and read segregation, each mRNA sample was aligned using tophat (version 2.0.0) (Trapnell et al. 2009), with default parameters against the reference *DrosophilaMelanogaster* genome and transcriptome (dm3/BDGP Release 5, from UCSC genome browser) (Adams et al. 2000; Fujita et al. 2011). Next, cuffdiff in the cufflinks package was used to determine differentially expressed genes and transcripts between the IPC replicates and the control replicates (Trapnell et al. 2010), where Reads Per Kilobase of exon model per Million mapped reads (RPKM) was calculated for each gene/transcript in each cell type and compared with each other.

**DAVID functional annotation analysis**

Functional annotation of 193 IPC-enriched transcripts was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang da et al. 2009b; Huang da et al. 2009a). Entrez_IDs of the 193 genes were used as input. 191 of 193 Entrez_IDs were annotated as known *Drosophila* genes by DAVID. Among the 191 known *Drosophila* genes, 115 (60.2%) genes have
biological function annotations in the GOTERM_BP_FAT database. Annotation categories were searched and clustered using GOTERM_BP_FAT databases. Biological functional clusters were ranked based on enrichment score, which was statistically measured by Fisher Exact in DAVID.

**Searching mouse homologs of fly genes**

Mouse homologs of IPC-enriched genes were found in NCBI HomoloGene Database Release 66 (http://www.ncbi.nlm.nih.gov/homologene/). This method identified mouse orthologs of 104 IPC-enriched genes (193 in total). In addition, 5 homolog pairs (Ilp2-Ins1, Ilp3-Ins1, Ilp5-Ins2, ia2-IA2, foxo-Foxo1) were added based on literature information (Brogiolo *et al.* 2001) and BLAT (UCSC genome browser) homologous sequence search.

**Fly tissue fixation and immunofluorescence**

Fly brains or fat bodies were dissected in ice-cold PBS containing 0.1% Triton X-100 (PBST) and tissues (brains or fat bodies) were fixed in 4% paraformaldehyde for 20 minutes, followed by extensive washes with PBST. 5% normal goat serum (NGS) (Sigma, diluted in PBST) was added to fixed samples for 1 hr at room temperature for blocking. Subsequently after washing out the NGS, primary antibodies (rat anti-ILP2 and rabbit anti-Foxo, Pierre Leopold; chick anti-GFP, Aves Labs; rabbit anti-mCherry/mtdTomato/dsRed, Clontech; rabbit anti-beta galactosidase, MP Biomedicals) were diluted in 5% NGS and incubated with the samples at 4°C overnight. After extensive washes with PBST, secondary antibodies (anti-rat Alexa 488, anti-rat Alexa633, anti-chick Alexa 488, and anti-rabbit Alexa 647, Invitrogen) were diluted in 5% NGS and incubated with samples at 4°C overnight. After PBST washes, samples were mounted in SlowFade Gold antifade reagent (Invitrogen) for slide preparation.

**Fly weighing pupae volume measurement**

Embryos were collected on a molasses cap within a 4 hr period followed by 24 hr incubation at 25°C. 30 newly hatched first instar larvae were then transferred to each vial and stayed at 29°C till eclosion. Adult males (1-2 day after eclosion) from each vial (typically in groups of 10 to 20) were weighed on an analytic balance accurate to ± 0.01 mg (Mettler Toledo). In the cases where balancer
exists for the UAS-RNAi lines or few adults emerged from each vial, males from multiple vials were combined for weighing.

Pupae were photographed. Pupae length (L) and diameter (D) were measured using ImageJ. *Drosophila* pupae are assumed to take the shape of an ellipsoid and its volume could be estimated based on this shape. Pupae volume (V) was calculated using the formula: $V = \frac{4}{3} \times 3.14159 \times \frac{D}{2}^2 \times \frac{L}{2}$.

**Trehalose and glucose measurement**

Trehalose assay was performed as previously described (Rulifson et al. 2002). In brief, hemolymph from 5-8 larvae was collected by tearing the cuticles and allowing the hemolymph to bleed out and pool on a siliconized glass slide. 0.5 µL of hemolymph samples as well as trehalose and glucose standards were diluted in 100 µL of Infinity Glucose Reagent (Sigma), and 40 µL of the diluted hemolymph was mixed with 80 µL of Glucose Infinity Reagent with porcine trehalase added at 3 µL/mL in 96-well plates. After 16-18 hrs incubation at 37°C, plates were read on a fluorescence plate reader (Bio-Tek Instruments model# FL600). Excitation was applied at 360 nm while emission occurred at 460 nm. Standard curves were generated for determining the combined glucose and trehalose concentration in hemolymph samples.

**Quantitative RT-PCR**

For each genotype, 3x10 third instar larval brains were collected and RNA was purified using RNAsy kit (Qiagen) according to manufacturer’s instructions. RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized from 1µg RNA using high capacity cDNA reverse transcription kit (Applied Biosystems). qPCR reactions for each biological sample were carried out in duplicate using Taqman assays (Applied Biosystems) for *Ilp2* and *Rpl32*. mRNA levels were compared between conditions using the $\Delta\Delta$Ct method.

**Confocal microscopy and image quantifications**

Fly and pupae images were captured using a Leica MZ FLIII fluorescent dissecting microscope and a Leica DFC 500 camera. Images were acquired using a TCS SP2 confocal microscope (Leica) with 63x/NA 1.4 oil-immersion objective. To quantify ILP2 levels, confocal Z-series of the IPCs were
obtained at 1µm step size with identical laser power and scanning settings between control and experimental samples. Image stacks were then projected into a Z-stack (sum intensity) and the mean fluorescence intensities across IPC cell bodies or fat body nuclei were measured using Image J (National Institutes of Health). For each larva, multiple brain IPCs were quantified and averaged. These averaged values were then used for statistics to estimate the variance between animals under the same genetic background. Error bars represent the standard error of the mean from at least three independent experiments.

Statistics

For fly weighing, pupae volume, trehalose level, IPC cell number, ILP fluorescence intensity, and qPCR results, data were represented as mean ± S.E.M. Student’s t-tests (two tailed, equal variance) were performed for statistical significance.
Figure Legends

Figure 1. IPC morphology changes during development. IPC neuronal structures were followed from early larval to adult stages. Developmental time points after embryo deposition were indicated. Green: Ilp2>mCD8-GFP; Red: Ilp2>nuclear-RFP; blue: DAPI. Up: Anterior; Down: Posterior. Shown are maximum z-projections of confocal images taken at 1µm step size. Flies were raised at 25°C throughout development. Scale bar: 50µm.

Figure 2. Capture of IPCs through laser-microdissection.
A. Drosophila IPCs viewed by confocal microscopy. Green depicts IPC neuronal processes and cell bodies and IPC cell nuclei are in red. B. Schematic describing the cryosections that were chosen for IPC laser-capture. C. GFP-labeled IPCs before and after laser capture. Red boxes outline where IPCs are located and insets show higher magnifications of those regions. Dotted lines demarcate the boundary of the brain. Scale bars: 50µm.

Figure 3. mRNA sequencing of IPCs and control neurons. A. 3’ bias of mRNA sequencing reads. Distribution of mRNA-seq reads along Ilp3 mRNA as seen in the UCSC Genome Browser (Karolchik et al. 2003). RefSeq Gene track shows Ilp3 mRNA structure. Wide blue bar: exons. Narrow blue bar: 5’ and 3’ untranslated region. Blue line: intron. Distribution of mRNA reads from IPC1, IPC2, Control1, and Control2 samples are showed as black bars in individual tracks. B. RT-PCR validation of laser-captured IPCs. Ilp2 and Ilp5 mRNA levels were assayed in amplified mRNAs from captured IPCs and control neural tissues. The housekeeping gene Ribosomal protein L32 (RpL32) was used as control. C. Scatter plot of the mRNA-Seq expression data of IPCs and controls. Both X- and Y-axis represent RPKM in log10 ratio. Red dots highlight IPC-enriched genes. Pseudo-count 0.01 were added to RPKMs of all genes to avoid errors in log-transformation of true. Thus, zero expression genes were represented as dots with expression of -2 in log10 on the plot. D. Histograms of RPKM levels of genes expressed in Controls and IPCs. x-axis is log10 scale of gene expression measured in RPKM, y axis is gene counts in log10 scale. Pseudo-count 0.01 were added to RPKMs of all genes to avoid errors in log-transformation of true. The dashed red line represents RPKM=5.
Figure 4. ILP2 is transported in DCVs along the axonal bundles in IPCs.

A. IPC neuronal structure from a third-instar larval brain was labeled with *Ilp2>*mCD8-GFP. ILP2 localizes to some IPC neuronal projections (white arrow) but not the others (yellow arrows). B-D. Localization of axonal (Tau) and dendritic (Khc::nod) markers in IPCs. In both figures, anterior (labeled with A) is towards the top and posterior (P) is towards the bottom. Arrowhead in B labels the portion of IPC axon bundles extending posteriorly from the cell bodies, and white arrow indicates after the turn, IPC axons projecting anteriorly. Yellow arrows in C indicate the dendritic branches of IPCs. D. schematic of IPC polarity. E. Localization of DCV in IPCs was examined by expressing exogenous ANF-GFP. Higher magnification of boxed region was shown for both ANF-GFP and ILP2 (single z optical section). Scale bars: 50µm except in the zoomed in images in E, which represents 10µm.

Figure 5. Unc-104 is localized to axons of IPCs and is required for IPC development and ILP2 secretion.

A. Control (*Ilp2>*w^1118, VDRC GD line control, #60000) and *Ilp2>*unc-104 RNAi (VDRC line#23465) third-instar brains were dissected. *Ilp2>*mCD8-GFP (green) indicates the whole IPC neural structure. B-C. ILP2 fluorescence intensities in the brain IPC cell bodies were compared between control (tubGal80^ts, *Ilp2>*w^1118, VDRC GD line control, #60000, n = 9 brains) and *unc-104* knockdown (n = 9 brains) in IPCs for 24hrs (tubGal80^ts, *Ilp2>*unc-104 RNAi). The images were taken with the same laser settings and manipulated in the same way. IPC cell bodies were outlined in the circle in control. D-E. Localization of Unc-104 in IPCs was examined by expressing exogenous *Unc-104-mCherry* or *Unc-104-GFP*. *Ilp2>*mCD8-GFP (green) indicates the whole IPC neural structure. Scale bars: 50µm. Error bars: S.E.M., **p<0.01.

Figure 6. Rab1 is required in IPCs for normal animal growth and development.

A-B. Adult weights and pupal volume were compared between control flies (*Ilp2*-Gal4>*yw, n = 153 flies, n= 5 pupae) and flies expressing *Rab1-DN* in IPCs (*Ilp2>*Rab1-DN, n = 138 flies, n = 6 pupae). C. In a single confocal optical section, IPCs were labeled with anti-ILP2 antibody and *Rab1* expressed cells were labeled with *Rab1>*Lamin-GFP. The other IPCs that were not displayed in this optical section also expressed *Rab1*. Scale bar: 20µm. D-E. Developmental time points of the onset of metamorphosis and
adult eclosion were compared between control flies and flies expressing \textit{Rab1-DN} in IPCs. \textbf{F.}
Combined trehalose and glucose level in the 3rd-instar larval hemolymph was compared between control (n = 12 pooled hemolymph samples) and \textit{Ilp2-Gal4>\textit{Rab1-DN}} flies (n = 10 pooled hemolymph samples). Data were represented as mean ± SEM. **p<0.01.

\textbf{Figure 7.} Rab1 colocalizes with ILP2 granules and is required for IPC development and ILP2 transport.
\textbf{A.} IPC neurite structure and nuclei were labeled with \textit{Ilp2>mCD8-GFP} and \textit{Ilp2>nls-RFP} in 3rd-instar larval brains. The white arrows in the top panel indicated the dendrites of control IPCs (\textit{Ilp2-Gal4>yw}), which were missing in \textit{Ilp2-Gal4>\textit{Rab1-DN}} brains. \textbf{B.} Quantification of IPC cell numbers (based on \textit{Ilp2>nls-RFP} labeled nuclei) indicated a reduction (5-6) in \textit{Ilp2-Gal4>\textit{Rab1-DN}} brains (n = 9 brains, for controls n = 10). \textbf{C-D.} ILP2 fluorescence intensities in the brain IPC cell bodies were compared between control (\textit{tubGal80ts, Ilp2>yw}, n = 10 brains) and Rab1 inhibition (n = 14 brains) in IPCs for 24hrs (\textit{tubGal80ts, Ilp2>\textit{Rab1-DN}}). The images were taken with the same laser settings and manipulated in the same way. \textbf{E.} Labeling of Rab1 (\textit{Ilp2>Rab1-YFP}), a Golgi marker (\textit{Ilp2>Grasp65-GFP}), or an endosome marker (\textit{Ilp2>FYVE-GFP}) in combination with ILP2 staining. The left column (lower magnification) shows the localization of GFP/YFP-tagged proteins as projection of z-stacks. Rab1 and FYVE-labeled endosomes were localized almost exclusively in the cell bodies, while Grasp65-labeled cis-Golgi distributed in both the IPC cell bodies and axons. Boxed regions are shown in higher magnification on the right columns. Rab1-YFP, Grasp-GFP, FYVE-GFP, and ILP2 all had punctate localization patterns in IPCs. For examination of colocalization between vesicles/granules, single z optical sections were shown. Scale bars: 50µm except in the zoomed in images in E, which represents 10µm. Error bars: S.E.M., **p<0.01.

\textbf{Table 1. Top ranked biological processes represented by IPC-enriched transcripts.} Each annotation cluster represents GO terms with similar biological functions determined by DAVID. Biological functional clusters were ranked based on the enrichment score. The top six GO terms that have the lowest \textit{p-values} (most enriched) within each cluster are showed in the table. All genes involved in each cluster are shown. Full list of the GO terms of each cluster is in Supplemental Table 5.
Supporting Information

Supplemental Movie 1. Movie of IPCs at different developmental stages. Green: Ilp2>mCD8-GFP; Red: Ilp2>nuclear-RFP; blue: DAPI.

Supplemental Movie 2. Rotating view of larval IPCs at 93 hrs after embryo deposition. Green: Ilp2>mCD8-GFP; Red: Ilp2>nuclear-RFP.

Supplemental Movie 3. Rotating view of pupal IPCs at 146 hrs after embryo deposition. Green: Ilp2>mCD8-GFP; Red: Ilp2>nuclear-RFP.

Supplemental Movie 4. Rotating view of adult IPCs at 239 hrs after embryo deposition. Green: Ilp2>mCD8-GFP; Red: Ilp2>nuclear-RFP.

Supplemental Figure 1. RNAi screen of IPC-enriched genes and Rab-DNs for growth phenotypes. A. 56 UAS-RNAi lines covering 50 genes were crossed to Ilp2-Gal4 and the progeny male adults were examined for their weights. These genes represent a wide range of molecular functions. They encode signaling molecules, transcription factors, neuropeptide receptors, motor proteins, sugar metabolic enzymes, synapse organizers, and etc. RNAi lines from different RNAi libraries are color-coded. Blue: Harvard TRiP lines; Green: VDRC GD lines; Orange: VDRC KK lines. The red-dashed line indicates the 90% adult weight cutoff. Error bars represent S.E.M. B. 43 UAS-Rab-DNs lines covering 29 Rabs were crossed to Ilp2-Gal4 and the progeny male adults were examined for their weight. Error bars represent S.E.M.

Supplemental Figure 2. Measurement of larval brain Ilp2 transcript levels when unc-104 mRNA was depleted or Rab1 protein function was inhibited. Ilp2 transcript levels were compared by quantitative RT-PCR between control and unc-104 knockdown in IPCs for 24 hrs (tubGal80°, Ilp2>unc-104 RNAi), or Rab1 inhibition in IPCs for 24 hrs (tubGal80°, Ilp2>Rab1-DN). Rpl32 was used as an internal control. N.S.: non significant. Error bars represent S.E.M.
Supplemental Figure 3. Expressing Rab1 RNAi in IPCs results in accumulation of ILP2 in IPCs.

A. Adult weights were compared between control flies (Ilp2-Gal4>attP2 control, n = 63 flies) and flies expressing Rab1 RNAi in IPCs (Ilp2>Rab1 RNAi, dicer2; n = 60 flies). B. IPC neurite structure was labeled with Ilp2>mCD8-GFP in Ilp2>Rab1 RNAi, dicer2 and control (Ilp2>dicer2, attP2 control) brains. C-D. ILP2 fluorescence intensities in the brain IPC cell bodies were compared between control (n = 8 brains) and Rab1 RNAi expressed in IPCs (n = 8 brains). Scale bars: 50µm. Error bar: S.E.M. **p<0.01.

Supplemental Table 1: Number of aligned mRNA reads to the D. Melanogaster Refseq mRNA.

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<th>Samples</th>
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Supplemental Table 2: List of all genes with aligned reads in laser-captured IPC and control samples.

Supplemental Table 3: List of 193 IPC-enriched genes with their annotated molecular functions.

Supplemental Table 4: Full list of biological functional clusters annotated by DAVID with IPC-enriched transcripts.

Supplemental Table 5: List of 109 IPC-enriched genes and their mouse orthologs. The mouse orthologs that have higher expression levels in beta cells compared to other non-beta cell tissues (Ku et al.) are indicated.
Supplemental Table 6: *Drosophila* stocks used in this study.

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<tr>
<th>Stock Description</th>
<th>UAS YFP.Rab2 DN</th>
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<td>w^{1118}</td>
<td>UAS YFP.Rab3 DN</td>
<td>UAS YFP.Rab26 DN</td>
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<td>UAS YFP.Rab27 DN</td>
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<td>UAS-GFP-myc-2xFYVE (Bloomington)</td>
<td>UAS YFP.Rab4 DN</td>
<td>UAS YFP.Rab30 DN</td>
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Supplemental Materials and Methods:

Detailed protocol for sequencing library construction

1) 400ng of amplified mRNA was fragmented to 10-200nt using 10x RNA fragmentation buffer (Ambion) and was purified using regular ethanol precipitation method with 0.35µl of GlycoBlue (Ambion).  

2) 3’ end the RNA samples were dephosphorylated using 10x Antarctic Phosphatase Buffer and 0.5 µl Antarctic Phosphatase (NEB) at 37 °C for 20 minutes. The reaction was heat inactivated at 75°C for 10 minutes.  

3) 5’ end of RNA samples was phosphorylated using 10x T4 DNA ligase buffer (it has 1mM ATP final) and T4 PNK (NEB) at 37 °C for 30 minutes. The RNAs in the reactions were purified using ammonium acetate and ethanol precipitation with 2µl of GlycoBlue (Ambion).  

4) The RNA samples are then ligated to 3’ linker (5’/-/5rApp/CTG TAG GCA CCA TCA AT/3ddC/-3’) (synthesized by IDT) using T4 RNA ligase 1 (NEB), 5X ATP-free T4 RNA ligase buffer (16.5 mM DTT, 41.5% glycerol, 250 mM HEPES-KOH, pH8.3, 50 mM MgCl₂, 50 µg/ml acetylated BSA), and 10% DMSO at 37 °C for one hour. The RNAs in the reactions were purified using ammonium acetate and ethanol precipitation with 2µl of GlycoBlue (Ambion). The RNA samples were then run on 6% TBE-Urea PAGE Gel (Invitrogen). The 100-200nt bands were cut and elute overnight with 400µl stop solution (1M ammonium acetate and 10mM EDTA) at 4°C overnight. The RNAs in the supernatant was purified using regular ethanol precipitation method with 2µl of GlycoBlue (Ambion).  

5) The RNA samples are ligated to 5’ linker (with bar code) using T4 RNA ligase 1 (NEB), 10x T4 RNA ligase 1 buffer (NEB), and 10% DMSO at 37°C for 1 hour. The RNAs was purified by ammonium acetate and ethanol precipitation and gel purification as described in step 4. The 5’ barcoded linkers are synthesized by IDT. IPC1: 5’/-/5AmMC6/ ACG CTC TTC CGA TCT rCrUrGrG-3’, IPC2: 5’/-/5AmMC6/ ACG CTC TTC CGA TCT rCrGrUrC-3’, Control 1: 5’/-/5AmMC6/ ACG CTC TTC CGA TCT rArCrUrU-3’, Control 2: 5’/-/5AmMC6/ ACG CTC TTC CGA TCT rCrCrCrU-3’.  

6) cDNA of the RNA samples were reverse transcribed using SuperScript III (Invitrogen) following manufacture’s protocol. The primer sequence used for reverse transcription is 5’-ATT GAT GGT GCC TAG TAC AG-3’.  

7) The cDNA samples were amplified using Taq (NEB) following manufacture’s protocol. Forward primer: 5’-GAT ACC ACC GAG ATC TAC ACT TCT TCC CTA CAC GAC GCT CTT CCG ATC T-3’. Reverse primer: 5’-CAA GCA GAA GAC GGC ATA CGA GCT CTT CCG ATC TAT TGA TGG TGC CTA
CAG-3’. The PCR products (200-300nt) were purified using Qiagen PCR purification kit. The purified
PCR samples were diluted to 10nM and were sequenced using Illumina GA II sequencing system.
Acknowledgements

We thank the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947), Vienna Drosophila Research Center, and Bloomington Stock Center for providing transgenic RNAi and other fly stocks used in this study. We appreciate generous help from Pierre Leopold, Eric Rulifson, Ping Shen, Tom Schwarz, Bill Saxton, Robin Hiesinger, and Liqun Luo, who provided flies and antibodies; Liqun Luo for supporting M.L.S and Ziming Weng, Phil Lacrout, and Arend Sidow for flow cell preparation, sequencing, and data processing; Sam Gu for help and suggestions on preparing mRNA-seq libraries for sequencing; and Virginia Walbot for sharing with us the cryostat and LCM in her lab. We also thank Liqun Luo, Tom Hartl, Ljiljana Milenkovic, and Alex Brown for critical comments on the manuscript. Research in the Kim lab was supported by Howard Hughes Medical Institute (HHMI). J.C. was supported by Stanford University’s Bio-X Interdisciplinary Initiatives Seed Grant. J.N. is a postdoctoral fellow, and S.K.K. and M.P.S. are both Investigators, of the HHMI.


Dong, H., M. Kumar, Y. Zhang, A. Gyulkhandanyan, Y. Y. Xiang et al., 2006 Gamma-aminobutyric acid up- and downregulates insulin secretion from beta cells in concert with changes in glucose concentration. Diabetologia 49: 697-705.


Wang, H., R. Ishizaki, J. Xu, K. Kasai, E. Kobayashi et al., 2013 The Rab27a effector exophilin7 promotes fusion of secretory granules that have not been docked to the plasma membrane. Molecular biology of the cell 24: 319-330.


Figure 4

A. Ilp2>mCD8-GFP  ILP2  Merged

B. Ilp2>mCD8-GFP  Ilp2>Tau-LacZ  Merged

C. Ilp2>mCD8-GFP  Ilp2>Khc::nod-LacZ  Merged

D. Axons  Dendrites

E. Ilp2>ANF-GFP  ANF  ILP2  Merged
### Table 1

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