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Drosophila Cyclin D/Cdk4 Requires Hif-1 Prolyl Hydroxylase to Drive Cell Growth

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Summary

The *Drosophila* cyclin-dependent protein kinase complex Cyclin D/Cdk4 induces cell growth (accumulation of mass) as well as proliferation (cell cycle progression). To understand how CycD/Cdk4 promotes growth, we performed a screen for modifiers of CycD/Cdk4-driven overgrowth in the eye. Loss-of-function mutations in Hif-1 prolyl hydroxylase (Hph), an enzyme involved in the cellular response to hypoxic stress, dominantly suppress the growth but not the proliferation function of CycD/Cdk4. *hph* mutant cells are defective for growth, and, remarkably, ectopic expression of Hph is sufficient to increase cellular growth. Epistasis analysis places Hph downstream of CycD/Cdk4. Overexpressed CycD/Cdk4 causes an increase in Hph protein in tissues where Hph induces growth, suggesting a mechanism whereby Hph levels are regulated posttranscriptionally in response to CycD/Cdk4. Our data suggest that Hph, in addition to its function in hypoxic response, is a regulator of cellular growth and that it is a key mediator for CycD/Cdk4.

Introduction

Patterning genes define tissue and body size through the regulation of cellular growth (accumulation of mass), cell proliferation (cell cycle progression), and cell death. Although growth and proliferation are often linked, they can be separated. Experiments from yeast and *Drosophila* demonstrate that growth is required for normal proliferation (Johnston et al., 1977; Neufeld et al., 1998). For example, when *Drosophila* wing disc cells are induced to proliferate faster than they normally do, they become smaller because division is faster than their accumulation of mass, which remains unchanged (Neufeld et al., 1998). Therefore, in order to understand size control, we must understand how growth, as well as proliferation, is regulated.

In *Drosophila melanogaster*, several pathways that regulate growth have been characterized over the last few years, including the cyclin-dependent protein kinase Cdk4/CycD (Finley et al., 1996; Sauer et al., 1996; Meyer et al., 2000; Datar et al., 2000). Flies homozygous mutant for *cdk4* are viable but are smaller in size, and their cellular growth rates are decreased (Meyer et al., 2000). Although vertebrate cyclin-dependent protein kinases Cdk4 or Cdk6 and associated cyclins (D1, D2, or D3) have been characterized mainly for cell cycle progression, their possible function in growth has not been extensively addressed. Mechanistically, vertebrate cyclin D/Cdk4-6 complexes induce cell cycle progression by phosphorylating and inhibiting pocket proteins including pRb, which are inhibitors of G1 to S phase progression (Adams, 2001). Rbf1, a pRb ortholog of *Drosophila* that was proposed to function downstream of CycD/Cdk4 (Duman-Scheel et al., 2002), can negatively regulate cell cycle progression when overexpressed (Du et al., 1996; Neufeld et al., 1998) but does not drive overgrowth when deleted (Datar et al., 2000). Therefore, we assumed that there must be other targets for CycD/Cdk4 that induce cellular growth.

Besides CycD/Cdk4, the growth drivers best characterized in *Drosophila* are components of the insulin signaling pathway and homologs of Myc and Ras oncogenes. When expressed in wing imaginal discs, all these factors cause cells to increase in size and shorten the G1 phase of the cell cycle. These factors do not, however, accelerate overall rates of cell cycle progression. Thus, proliferation rates are at least partially uncoupled from growth. This induction of growth is phenotypically different from that caused by CycD/Cdk4, where growth leads to an increase in cell cycle progression. Therefore, under CycD/Cdk4, proliferation is linked to growth.

Several other drivers characterized in *Drosophila* show a similar induction of growth and proliferation, including *bantam*, members of the Hedgehog (Hh) signaling pathway, and mutants of the Salvador/Warts/Hippo complex (Hay and Guo, 2003). *bantam*, a microRNA, was proposed to regulate protein translation of yet unidentified growth targets (Brennecke et al., 2003). However, *bantam* seems to function independently from CycD/Cdk4 and, unlike CycD/Cdk4, regulates cell death (Hipfner et al., 2002). In contrast, Hh signaling requires Cdk4 to drive cellular growth (Duman-Scheel et al., 2002). Although these data put CycD/Cdk4 downstream of Hh, it is still unknown how CycD/Cdk4 drives growth.

In addition to genetic factors, extrinsic factors like nutrients, temperature, or oxygen concentrations determine the size of insects. *Drosophila* reared at hypoxic O_2 concentrations (lower than the normal 21%) develop into proportionally normal but smaller adults and hence have a decreased growth rate (Palos and Blasko, 1979; Frazier et al., 2001). Larvae exposed to hypoxia experience an arrest of cell cycle progression and show a so-called "rowing" phenotype, presumably to escape hypoxic conditions found, for example, in fermenting fruit (DiGregorio et al., 2001; Douglas et al., 2001). On the cellular level, hypoxia leads to the stabilization of a PAS domain, basic helix-loop-helix transcription factor, the heterodimer of Sima/Hif-1α and Tango/Hif-1β (Nambu et al., 1996; Bacon et al., 1998; Lavista-Llanos et al., 2002; Ma and Haddad, 1999; Sonnenfeld et al., 1997). Vertebrate Hif-1α/β is a key mediator of the cellular response to low oxygen levels. It binds to a consensus sequence in promoters called HRE (hypoxia responsible element), and its target genes regulate glucose and iron

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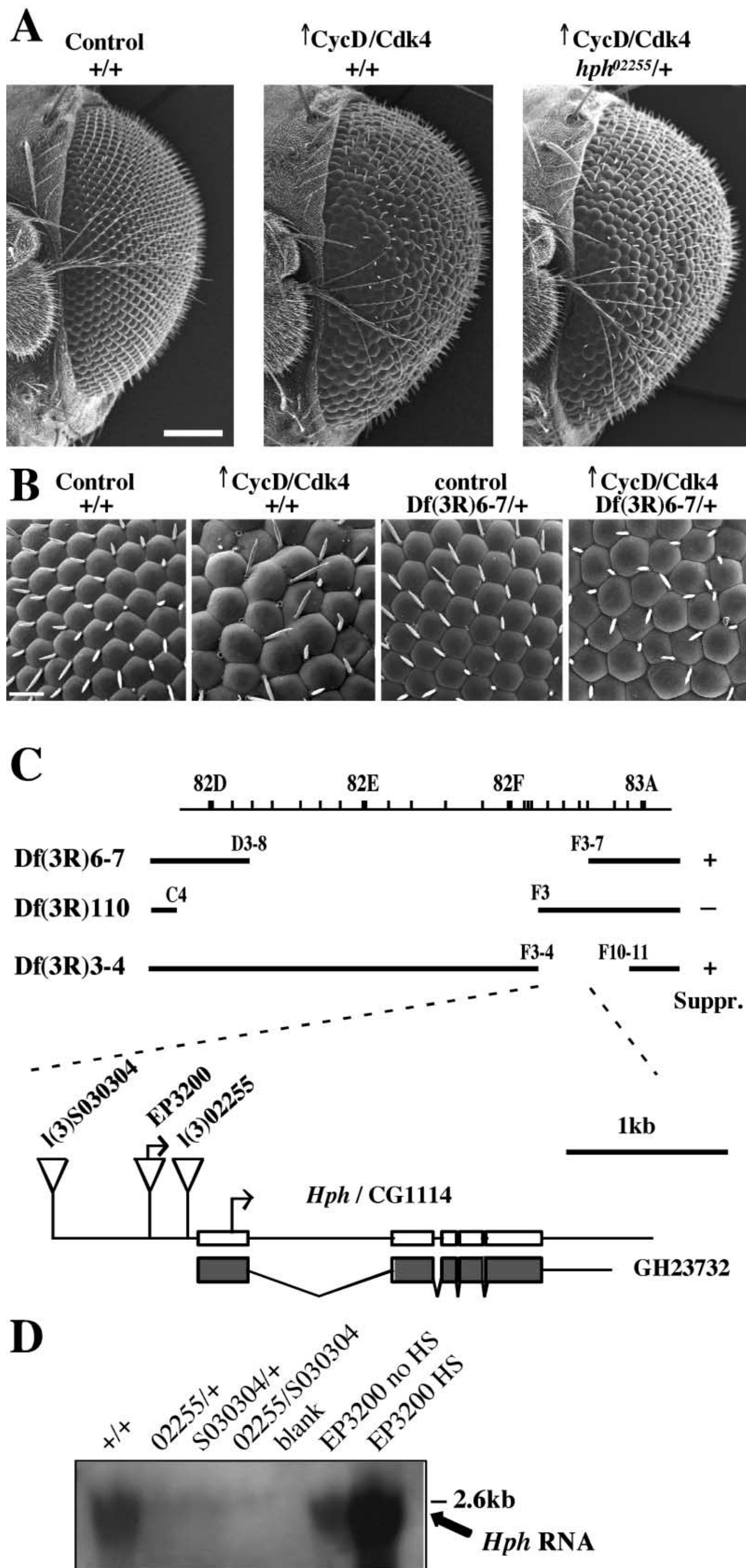

Figure 1. *hph* Mutants Suppress the Overgrown Eye Phenotype of CycD/Cdk4

(A) Scanning electron micrograph (SEM) images at 90× magnification of control flies (GMR-Gal4; left), expressing GMR-Gal4-driven CycD/Cdk4 in wild-type (middle) and hph^{02255}/+ (right) backgrounds. Scale bar, 100 μm.

(B) Side view with GMR-Gal4-driven CycD/Cdk4 in Df(3R)6-7/+ background at 500× magnification. Scale bar, 20 μm.

(C) Deficiencies identified as modifiers of CycD/Cdk4-driven overgrowth. Df(3R)6-7 and Df(3R)3-4 suppress the overgrowth phenotype but not Df(3R)110. (Bottom) Genomic locus of Hph/CG1114. l(3)02255 is inserted 104 bp upstream of putative transcription start site of Hph, EP3200 382 bp, and l(3)S030304 1111 bp, respectively. The full-length cDNA clone (GH23732) that was used to generate UAS-Hph is shown at the bottom (filled gray).

(D) Northern blot for *Hph*. Total RNA was prepared form third instar larvae and probed for *Hph* using in vitro transcribed RNA from the Sp6 promoter of GH23732 (see Experimental Procedures).

metabolism, angiogenesis, and possibly cell cycle progression (Kaelin, 2002; Semenza, 2001; Masson and Ratcliffe, 2003; Wenger, 2002). Whereas Hif-1β levels are unchanged and not rate limiting, Hif-1α protein levels are highly regulated. Under normal oxygen conditions (normoxia), Hif-1α is hydroxylated on conserved proline

residues by members of the HPH/EGLN/PHD protein families (Bruick and McKnight, 2001; Epstein et al., 2001). The reaction requires the binding of Fe^{II} as well as O_2, linking the amount of hydroxylation directly to the concentration of intracellular oxygen. Once hydroxylated, Hif-1α is recognized by an E3 ligase complex and mediated for ubiquitin-dependent protein degradation. Therefore, Hif-1α can only accumulate in the absence of oxygen. Besides the hydroxylation by Hph, Hif-1α is modified and regulated in various manners, although these effects seem to be less important and might reflect fine tuning (Masson and Ratcliffe, 2003; Kaelin, 2002; Wenger, 2002). These include the nuclear localization, DNA binding, and transactivation activity of Hif-1α/β. Importantly, a direct connection between Hif-1α/β and growth has not been addressed properly, and the molecular mechanism that links growth rates with oxygen concentrations is still unclear.

In order to identify gene products required for CycD/Cdk4-driven growth, we performed genetic loss-of-function screens to identify mutants that function downstream of CycD/Cdk4. This manuscript describes the characterization of one of these mutants, *hph*, which was previously identified as a regulator of cellular hypoxia.

Results

hph Suppresses CycD/Cdk4 in the Eye

GMR-Gal4 is expressed in the eye imaginal disc posterior to the morphogenetic furrow, where only cells in the second mitotic wave undergo one synchronized cell division. Therefore, UAS transgenes driven by GMR-Gal4 are expressed predominantly in postmitotic cells. Under these circumstances, expression of CycD/Cdk4 leads to an enlargement of the adult eye, bigger ommatidia and bristles, and a general rough appearance (Datar et al., 2000). Although some ommatidia have additional cells, the main cause of the enlargement is an increase in cell size leading to 61% larger ommatidia (Figure 1B). In order to identify loss-of-function mutants that modify this phenotype, CycD/Cdk4 was expressed in a deficiency collection background and screened under the light microscope for modifiers. Out of 162 deficiencies that cover 60%–70% of the genome, four deficiencies were isolated that dominantly suppressed CycD/Cdk4. Df(3R)6-7, which deletes polytene segments 82D3/8-F3/6, led to a decrease in the enlargement of the eye and bristle size. Most strikingly, under these conditions, CycD/Cdk4 led to an increase in ommatidia size of only 17% (Figure 1B). Subsequently, partial overlapping deficiencies were tested: Df(3R)3-4 showed the same suppression phenotype but not a third deficiency, Df(3R)110, demonstrating that the gene of interest is between 82F3 and F7 (Figure 1C). All available mutants in this region were tested, and two lethal P element insertions were identified that showed an identical suppression phenotype compared to the deficiencies: l(3)02255 is inserted 104 bp, and l(3)S030304 is inserted 1111 bp upstream of the putative transcription start site of Hph/dmHph/CG1114 (Figures 1A and 1C and Experimental Procedures).

We next addressed whether l(3)02255 and l(3)S030304 are alleles of *hph* and whether their loss of function causes the suppression of CycD/Cdk4. Northern blot experiments revealed that both heterozygous mutants l(3)02255 and l(3)S030304 have reduced expression of *hph* compared to the endogenous levels and that l(3)02255/l(3)S030304 transheterozygotes lack all detectable *hph* expression (Figure 1D). In addition to these loss-of-function mutants, the EP3200 line has an EP insertion 382 bp upstream of Hph. Expression of *hph* using this EP element, by the hs-Flp Act>CD2>Gal4 system, led to a weak expression of *hph* in the absence of a heat shock, due to leakage of the system. A further increase in *hph* expression occured upon heat shock (Figure 1D).

A full-length Hph cDNA was cloned under the control of a UAS promoter and injected into flies (Figure 1C; see Experimental Procedures). These Hph transgenes suppress at least partially the cell growth phenotype of homozygous *hph* mutants (Figure 4B) and completely suppress the reduced viability of l(3)02255/l(3)S030304 transheterozygotes (Experimental Procedures). Furthermore, when UAS-Hph transgenes were coexpressed with CycD/Cdk4 using the GMR-Gal4 driver, the dominant suppression of CycD/Cdk4-driven overgrowth by l(3)02255 was inhibited and the overgrowth phenotype was restored (data not shown). We conclude that l(3)02255 and l(3)S030304 are alleles of Hph and that the reduction of Hph can suppress CycD/Cdk4-induced overgrowth.

hph^{02255} Suppresses CycD/Cdk4-Driven Cell Enlargement in the Eye Imaginal Disc

We next tested whether growth or proliferation would be suppressed by *hph* in the eye imaginal disc. GFP was expressed either alone or together with CycD/Cdk4 in wild-type, $hph^{02255}/+$, or Df(3R)3-4/+ backgrounds using the GMR-Gal4 driver. Imaginal eye discs from wandering third instar larvae were dissected, trypsinized to single cells, and analyzed by FACS for their cell size by using the forward scatter (FCS). Figure 2A shows posterior cells overexpressing CycD/Cdk4 and GFP in the different backgrounds, indicated by black lines. Control posterior cell populations, expressing GFP alone, are indicated with filled gray histograms. Expression of CycD/Cdk4 in a wild-type background led to an increase in the forward scatter of 20%–30% (Figure 2A, left), which was reduced to 10%–15% in an $hph^{02255}/+$ or Df(3R)3-4/+ background (Figure 2A, middle and right). Acridine orange staining showed that the suppression phenotype was not due to an increase in cell death (data not shown). Furthermore, the cell cycle distribution was analyzed of eye imaginal discs from wandering larvae or pupae 48 hr after prepupae formation. At both time points, the increase in cells entering S and G2/M phases of the cell cycle due to ectopic expression of CycD/Cdk4 was not altered in an $hph^{02255}/+$ background (data not shown; Figure 2B). Taken together, these data demonstrate that the cell size and proliferation functions of CycD/Cdk4 can be separated. Furthermore, Hph is required for the increase in cell size but not required for proliferation, suggesting that Hph functions downstream of CycD/Cdk4 in a growth-specific manner.

hph^{02255} Suppresses the Growth Phenotype of CycD/Cdk4 in the Wing Imaginal Disc

In the experiments shown above, CycD/Cdk4 was induced in mostly postmitotic cells of the eye imaginal

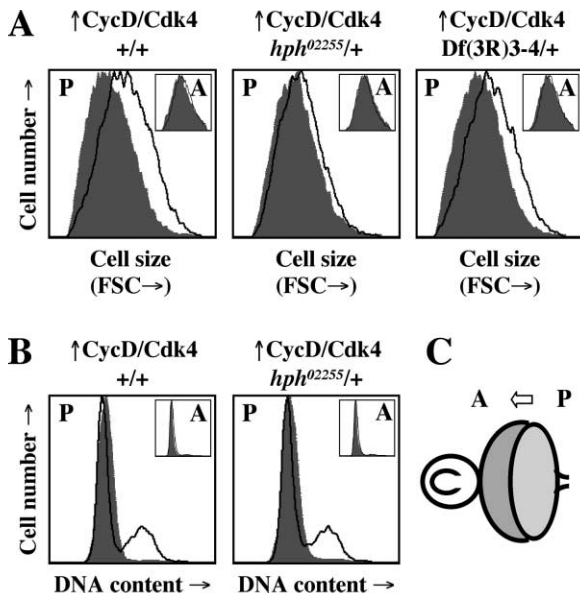

Figure 2. Heterozygous *hph* Mutants Suppress the Cell Size Phenotype of GMR-Gal4-Driven CycD/Cdk4 in the Eye Imaginal Disc

(A) FACS of eye imaginal discs from third instar larvae. The foreword scatter (x axis) indicative of the cell size is blotted against the number of cells (y axis). The black line represents posterior cells expressing CycD/Cdk4 together with GFP compared to posterior expressing only GFP (filled gray). Genetic backgrounds are indicated on top. Insets are anterior cells.

(B) FACS of pupal eye imaginal discs 48 hr after white prepupae formation. DNA content (x axis) is plotted against cell number (y axis). Note: GFP-negative cells are non eye disc cells. Genotypes in (A) and (B) in the indicated backgrounds: gray filled, posterior cells GMR-Gal4/+; UAS-GFP/+; black line, posterior cells GMR-Gal4/UAS-CycD UAS-Cdk4; UAS-GFP/+.

(C) Drawing of third instar eye and antenna imaginal discs showing the morphogenetic furrow (arrow) moving from posterior to anterior.

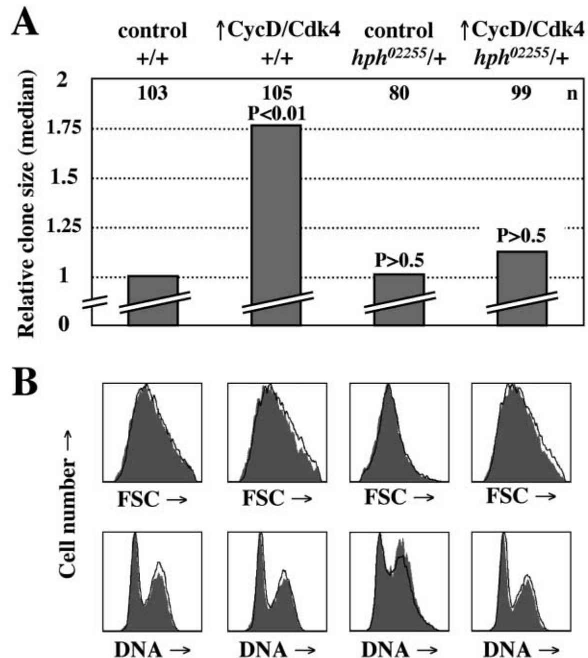

Figure 3. *hph*^{02255} Suppresses the Clone Size Phenotype of Ectopic CycD/Cdk4 in the Wing Imaginal Disc

(A) Clones expressing GFP or CycD/Cdk4 plus GFP in wild-type or *hph*^{02255}/+ background were induced and area measured as described in Experimental Procedures.

(B) FACS analysis of wing discs. Clones are induced as in (A). The black line represents cells overexpressing GFP plus CycD/Cdk4 (second from left and right) or GFP alone (left and third from left), and the gray filled histogram represents the GFP-negative cells (internal control). Genotypes: yw hs-Flp^{122}; +; Act>CD2>Gal4 UAS-GFP/+ (left), yw hs-Flp^{122}; UAS-CycD UAS-Cdk4/+; Act>CD2>Gal4 UAS-GFP/+ (second from left), yw hs-Flp^{122}; +/+; Act>CD2>Gal4 UAS-GFP/*hph*^{02255} (third from left) and yw hs-Flp^{122}; UAS-CycD UAS-Cdk4/+; Act>CD2>Gal4 UAS-GFP/*hph*^{02255} (right). Note: control in *hph*^{02255}/+ background was from a separate experiment with a separate control.

disc. To test suppression by *hph* in mitotically dividing cells, CycD/Cdk4 was induced during larval development, and wing discs cells were analyzed. Ectopic expression of CycD/Cdk4 shows a distinctive induction of growth: cells divide at a faster rate but are otherwise indistinguishable from control cells from the same disc. Therefore, when single clones are measured, the clone area is increased, and the clone consists of more cells with no change in cell size or cell cycle phasing (Datar et al., 2000). Since columnar cells of wing discs form a single cell layer, measuring the clone area gives an accurate estimation of the amount of mass that was accumulated during the growth of the clone. CycD/Cdk4 was overexpressed together with GFP in random clones using the hs-Flp Act>CD2>Gal4 system and analyzed after a 48 hr growth period. Compared to external control clones expressing only GFP, expression of CycD/Cdk4 caused a 75% increase in the median clone size. This phenotype depends on Hph, since the median clone size was reduced to control level in a heterozygous *hph*^{02255} mutant background (Figure 3A). The suppression did not correlate with an increase in apoptosis, since coexpression of the cell death inhibitor p35 gave identical phenotypes (data not shown). When cell size and cell cycle phasing were analyzed by FACS, there was no difference between cells expressing CycD/Cdk4 and internal control cells in either wild-type or *hph*/+ mutant backgrounds (Figure 3B). These results demonstrate that the induction of growth by CycD/Cdk4 depends on normal levels of Hph. Furthermore, since *hph* suppressed growth but not proliferation in the eye imaginal discs (Figure 2), expression of CycD/Cdk4 in the wing should lead to a change in cell size if only growth but not proliferation were suppressed. However, we did not detect a difference in cell size, suggesting that the increase in proliferation caused by CycD/Cdk4 is secondary to the induction of growth.

hph Mutants Are Defective for Growth

We next tested whether *hph* function was required for normal rates of cell growth. Most *hph*^{0225}/*hph*^{02255} or *hph*^{02255}/Df(3R)3-4 animals die during embryogenesis, and only a few larvae hatch. These mutant larvae have severe growth defects and die within 2 to 3 days (data not shown). Transheterozygotic *hph*^{S030304}/*hph*^{02255} mutants develop normally until pupariation, but very few escaper adults eclose. These escapers are smaller than

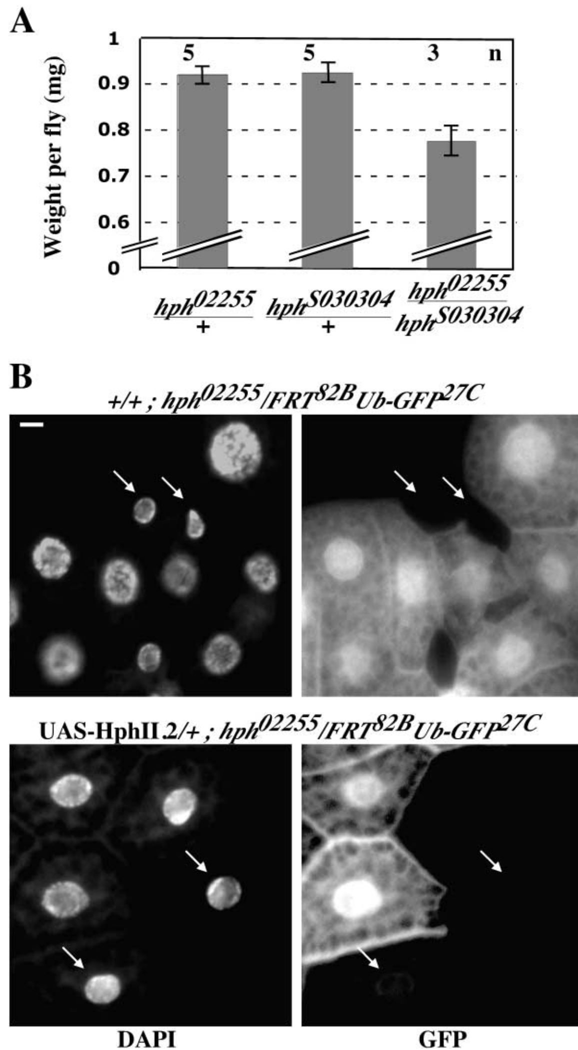

Figure 4. Homozygous *hph* Mutants Are Defective for Growth

(A) Flies were grown at 25°C and males were weighed 2 days after eclosion in batches of 20.

(B) hph^{02255}/hph^{02255} cells were induced by radiation (1200 rad) during embryogenesis. Fat bodies from third instar larvae were dissected, mounted, and stained with DAPI for DNA. Genotypes: w; +/+; hph^{02255}/FRT82B Ub-GFP^{27C} (top) and w; UAS-HphII.2/+; hph^{02255}/FRT82B Ub-GFP^{27C} (bottom). Scale bar, 10 μm.

their heterozygous siblings but have normal body proportions. Weight measurements showed that $hph^{S030304}/hph^{02255}$ mutant flies are 18% lighter than heterozygotes (Figure 4A). Therefore, *hph* mutant animals show a phenotype similar to homozygous $cdk4^3$ flies (Meyer et al., 2000) or wild-type flies reared at low oxygen (Palos and Blasko, 1979; Frazier et al., 2001).

To test whether *hph* mutant cells are autonomously defective for growth, homozygous mutant clones were induced in the fat body using ionizing radiation. hph^{02255} was crossed to flies expressing GFP under the control of a constitutively active promoter inserted on the same arm of the chromosome as Hph (3R). The progeny were irradiated during embryogenesis, emerging larvae were grown in regular food, wandering third instar larvae were dissected, and their fat bodies were fixed and mounted. Homozygous hph^{02255} mutant cells lacked GFP, whereas heterozygous mutant cells expressed GFP. Figure 4B shows that hph^{02255}/hph^{02255} cells were smaller and contain less DNA than heterozygous neighboring cells (Figure 4B, top; arrows). Importantly, the presence of a UAS-Hph transgene partially suppressed this phenotype, indicating that loss of Hph was the cause of the growth defect (Figure 4B, bottom).

Expression of Hph Is Sufficient to Stimulate Growth

We next tested whether ectopic expression of Hph was sufficient to stimulate growth. We used EP3200 or UAS-Hph transgenes to induce Hph expression. Cell clones expressing Hph were induced in wing imaginal discs, and the median clone size was measured as in Figure 3. Expression of Hph led to an increase in clone area very similar to CycD/Cdk4 (Figure 5A). Surprisingly, expression of Hph together with CycD/Cdk4 stimulated clonal growth to the same extent as Hph alone. However, in the presence of the apoptosis inhibitor p35, we detected an additive phenotype when both growth drivers were coexpressed (data not shown). As seen above for CycD/Cdk4, overexpressed Hph did not change cell size or cell cycle phasing, as assayed by FACS (data not shown).

To test whether Hph functions downstream of CycD/Cdk4 also in this tissue, clones expressing Hph were induced in a homozygous $cdk4^3$ mutant background, and the median clone size was measured as above. Figure 5B shows that, under these conditions, Hph led to a very similar induction of growth as in a wild-type background. FACS analysis indicated that there were no detectable changes in cell size or cell cycle phasing (data not shown). These data show that Hph is sufficient to stimulate growth, and the finding that this stimulation is independent of Cdk4 suggests that Hph functions downstream of CycD/Cdk4.

Ectopic expression of CycD/Cdk4 in the posterior compartment of the wing imaginal disc using the en-Gal4 promoter leads to an enlargement of the posterior compartment in adult wings (Hipfner et al., 2002) with no change in trichome (hair) density (data not shown). Since the trichome density is proportional to the number of cells per area, the increase in compartment size is due to more cells of the same size. When Hph was expressed under en-Gal4 control using EP3200 (Figure 5C) or UAS-Hph transgenes (data not shown), a similar result was obtained: posterior compartments were bigger and contained more cells of the same size. Thus, Hph induces growth in a similar manner to CycD/Cdk4 in wing imaginal discs. However, when Hph was expressed in the eye imaginal disc using the GMR-Gal4 driver, we did not observe an increase in cell size, as assayed by FACS. Furthermore, adult eyes were not enlarged (data not shown). Further experiments are required to understand why Hph expression is not sufficient to increase growth in the eye imaginal disc.

Hph Protein Levels Are Regulated by CycD/Cdk4

Little is known about how Hph RNA or protein levels are regulated. In *Drosophila* embryos, *hph* is expressed

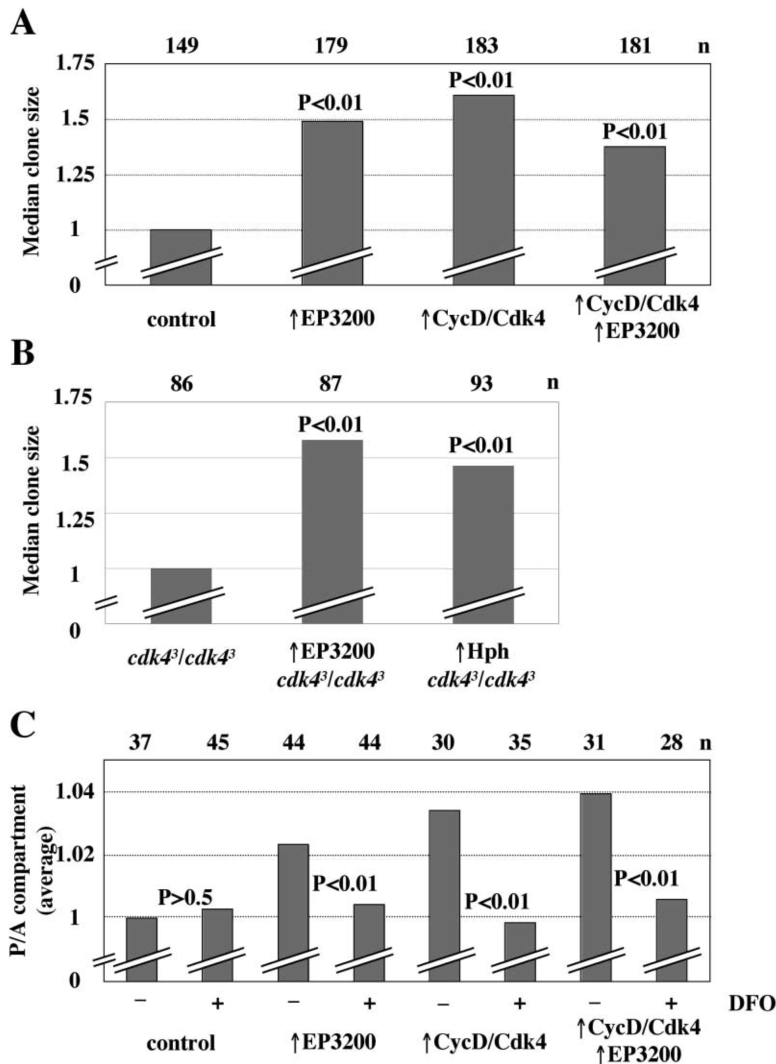

Figure 5. Ectopic Expression of EP3200/Hph Is Sufficient to Induce Growth

(A and B) Median clone size relative to control. Clones are induced and p values calculated as in Figure 3A. Genotypes: (A) yw hs-Flp^{122}; +; Act>CD2>Gal4 UAS-GFP/+ (left), yw hs-Flp^{122}; +; Act>CD2>Gal4 UAS-GFP/EP3200 (second from left), yw hs-Flp^{122}; UAS-CycD UAS-Cdk4/+; Act>CD2>Gal4 UAS-GFP/+ (third from left) and yw hs-Flp^{122}; UAS-CycD UAS-Cdk4/+; Act>CD2>Gal4 UAS-GFP/EP3200 (right); (B) yw hs-Flp^{122}; *cdk4*^{3}/*cdk4*^{3}; Act>CD2>Gal4 UAS-GFP/+ (left), yw hs-Flp^{122}; *cdk4*^{3}/*cdk4*^{3}; Act>CD2>Gal4 UAS-GFP/EP3200 (middle) and yw hs-Flp^{122}; *cdk4*^{3}/*cdk4*^{3}; Act>CD2>Gal4 UAS-GFP/UAS-HphIII.1.

(C) Transgenes are expressed using the posterior specific en-Gal4 driver. Flies were grown in normal food or supplemented with 2 mM DFO, and wings from adult males were mounted and the posterior and anterior compartment areas were measured. Values indicate the area of posterior compartments relative to anterior compartments, where the control without DFO was set to 1. p values are standard t test with equal variances for flies with DFO compared to without DFO.

uniformly and does not seem to be subject to patterning. Vertebrate cells have three Hph orthologs (Taylor, 2001): HPH-3 protein localizes to the nucleus, HPH-2 exclusively to the cytoplasm, and HPH-1 mainly in the cytoplasm with a little staining in the nucleus (Huang et al., 2002; Metzen et al., 2003).

To test whether the subcellular localization or levels of Hph are altered in response to CycD/Cdk4, we raised polyclonal antibodies to full-length Hph (see Experimental Procedures). To test the specificity of the antiserum, Hph was overexpressed in the posterior compartment of the wing using the en-Gal4 driver. Hph staining increased in posterior regions, both in the peripodial (Figure 6A) and columnar epithelium (Figure 6B). Homozygous *hph* mutant cells, marked by the absence of GFP (arrows), lacked detectable Hph staining (Figure 6C). Therefore, the serum is specific for Hph and is able to detect endogenous levels of Hph. Furthermore, in third instar imaginal wing discs, Hph staining was uniform throughout the disc and specific for the nucleoplasm of the cells. Very little staining was detectable in the cytoplasm or the nucleolus.

When Hph staining was analyzed in cells expressing ectopic CycD/Cdk4, increased Hph levels were observed. Antiserum staining in the peripodial epithelium showed that these cells had an increase in nucleoplasmic as well as cytoplasmic Hph (Figure 6D). In contrast, when homozygous *cdk4*^{3} mutant cells, marked by the absence of GFP, were analyzed, we detected only background levels (Figure 6E, arrows). Therefore, Hph protein levels are regulated in response to CycD/Cdk4 gain and loss of function. When *hph* expression was analyzed by RT-PCR from wing discs expressing ectopic CycD/Cdk4, we did not detect an effect on Hph RNA levels (Figure 6F). Furthermore, when whole third instar larvae were analyzed by RT-PCR or microarray analysis, no change in Hph expression was observed (data not shown; B. Lynch and B.A.E., unpublished data). Taken together, these observations suggest that CycD/Cdk4 affects Hph levels posttranscriptionally.

The hydroxylation activity of HPHs depends of Fe^{2+} bound to the active site (Epstein et al., 2001). Therefore, iron chelators like deferoxamine mesylate (DFO) are commonly used to experimentally mimic hypoxic conditions. When *Drosophila* larvae were raised on regular food supplemented with 2 mM DFO, they showed an induction in Hif-1α/β, as assayed with a reporter construct (Lavista-Llanos et al., 2002), that was very similar to that seen under hypoxic conditions (data not shown). When CycD/Cdl4 or Hph were expressed in the posterior

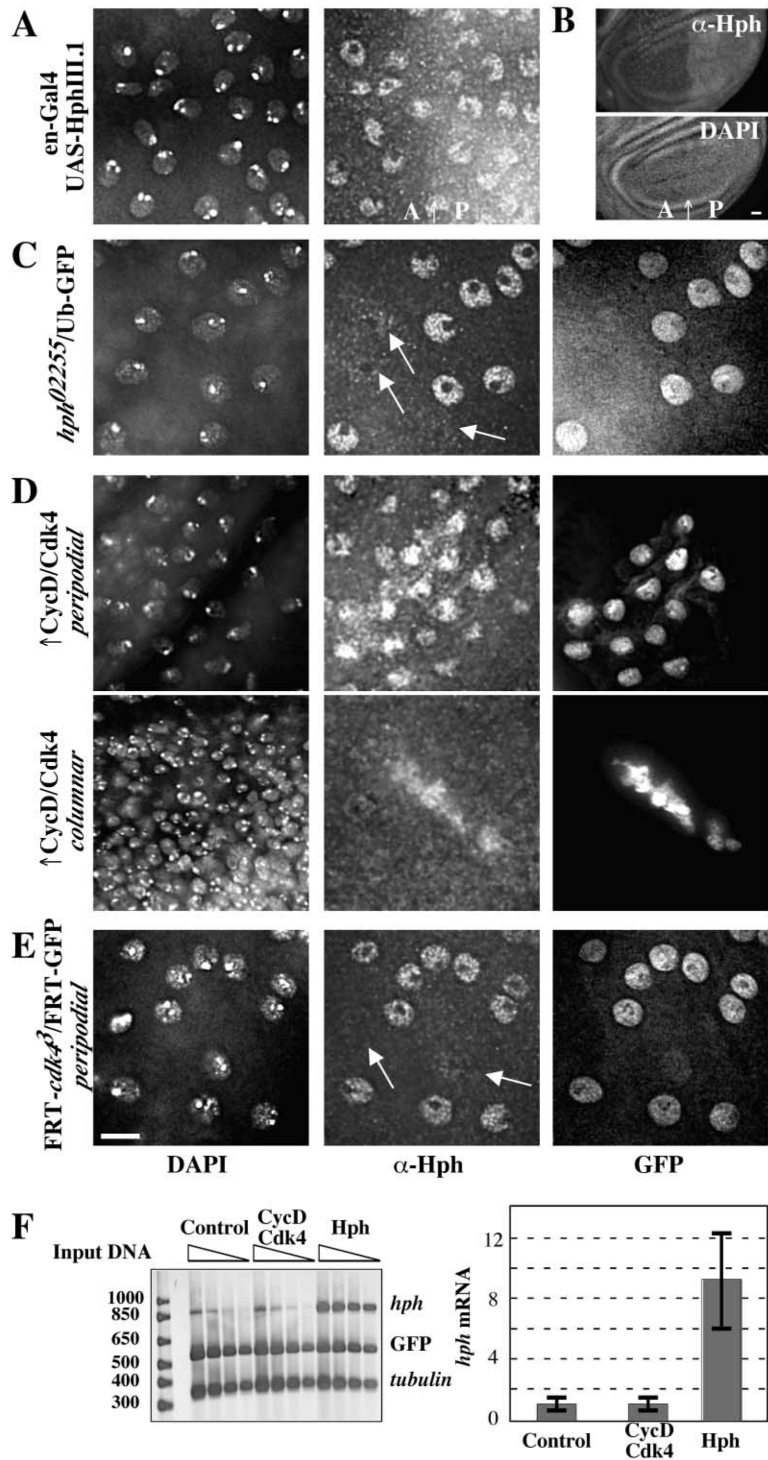

Figure 6. CycD/Cdk4 Regulates Hph Protein Levels

(A–E) Polyclonal staining for Hph in wing imaginal discs from larvae at 114 hr AED.

(A) UAS-HphIII.1 was expressed using the en-Gal4 driver. Shown is the anterior/posterior compartment boundary in the peripodial epithelium above the wing pouch.

(B) Lower magnification of (A) showing the wing pouch.

(C) *hph*^{02255}/Ub-GFP animals were irradiated at 66 hr AED to induce homozygous mutant *hph* cells as in Figure 5B (white arrows).

(D) CycD/Cdk4 was expressed in random clones using the Flp/Act>CD2>Gal4 UAS-GFP method. Larvae were heat shocked at 66 hr AED. Clones expressing ectopic CycD/Cdk4 were identified by coexpression of GFP (right). Shown are cells from the peripodial (top) and columnar epithelia (bottom).

(E) Homozygous *cdk4*^{3} cells (arrows) were induced as described in Meyer et al. (2000). Scale bar, 10 μm.

(F) CycD/Cdk4 does not change *hph* expression by RT-PCR. Total RNA from 70 wing discs expressing CycD/Cdk4 or Hph (induced as in [D]) was isolated, reverse transcribed, and mRNA levels for *hph*, *tubulin*, and GFP determined by PCR (left). (Right) Signals from two different experiments and two gels each were quantified, and *hph* levels were normalized to *tubulin* and GFP.

compartment of the wing imaginal disc, the increase in compartment areas was suppressed by DFO (Figure 5C). Moreover, when DFO was added to flies expressing CycD/Cdk4 in the postmitotic eye using the GMR-Gal4 driver, the enlargement of the adult eye as well as the rough appearance was suppressed, however, not to the same extent as in the heterozygous *hph* mutant backgrounds (data not shown). This suggests that the hydroxylation activity of Hph is required for its growth function and that Hph is a major growth effector of CycD/Cdk4.

Discussion

Most cyclin-dependent protein kinases have been extensively characterized for functions in cell cycle progression. According to prevailing models, vertebrate Cdk4 or Cdk6, bound to cyclins D1, D2, or D3, function

in cell cycle progression during G1. These complexes initiate the inhibitory phosphorylation of pocket proteins of the pRb family, which are inhibitors of cell cycle progression (Adams, 2001). Mice deleted for Cdk4 or cyclin D1 do not have widespread proliferation phenotypes, and this has been attributed to the presence of redundant kinases (Cdk6) or the remaining D-type cyclins. However, one remarkable phenotype, very similar to flies deleted for *cdk4*, is the size of the animals: *Cdk4*$^{-/-}$ or cyclin *D1*$^{-/-}$ mice are small (Fantl et al., 1995; Tsutsui et al., 1999). At birth, these animals are about 20% smaller than control littermates, and subsequently they gain weight at a slower pace. These growth defects could be attributed to defects in cell cycle progression or, alternatively, to defective cellular growth.

In *Drosophila*, a compelling case has been made that CycD/Cdk4 promotes cellular growth as well as cell cycle progression (Meyer et al., 2000; Datar et al., 2000). Our finding that *Drosophila* Hph functions downstream of CycD/Cdk4 and is sufficient to increase growth when overexpressed suggests that CycD/Cdk4 and Hph work in a common pathway. Consistent with this, heterozygous *hph* mutants do not suppress the extra growth induced by components of the insulin pathway or dMyc (data not shown). Moreover, increases in cell size and changes in cell cycle phasing induced by the insulin signaling pathway, dMyc, or Ras in wing imaginal disc cells, do not depend on Cdk4 (data not shown). Taken together, these results suggest that the CycD/Cdk4-Hph pathway functions separately from these other growth regulatory pathways.

Since the kinase activity of Cdk4 is required for the induction of growth and proliferation (Meyer et al., 2000), Hph could be a phosphorylation target of Cdk4. The consensus sequence of vertebrate pocket proteins, the only known targets of cyclin D1/Cdk4, can be different from the classical CDK sequence (Kitagawa et al., 1996). In *Drosophila* Rbf1, two potential sites have been found that disrupt its regulation by CycD/Cdk4 and CycE/Cdk2: T^{356}PLTR and S^{728}PHPK (Xin et al., 2002). Both sites are different from the vertebrate consensus sequence. Therefore, a search for putative consensus sequences on Hph is difficult. However, there are three sites that have the minimal requirement of a serine or threonine residue followed by a proline: T^{91}PDAP, T^{204}PGTT, and T^{285}PPAA. None of these resemble the consensus sequences recognized by either the vertebrate or *Drosophila* complex. Nevertheless, future experiments should address whether CycD/Cdk4 phosphorylates Hph on these or other sites and how this affects Hph function.

We propose that in wing discs, Hph protein levels are regulated in response to CycD/Cdk4 (Figure 7). Although we cannot exclude that growth is also induced in an Hph-independent manner, the findings that overgrowth driven by CycD/Cdk4 and Hph is suppressed nearly completely by the iron chelator DFO (Figure 5C) or by heterozygosity for *hph* (Figure 3A) suggest that this is a major mechanism. Moreover, we propose that the small size of flies reared at low oxygen concentrations is caused at least partially by a decrease in Hph activity due to the absence of oxygen.

How does Hph induce growth? Since the hydroxylation inhibitor DFO suppressed the increases in growth

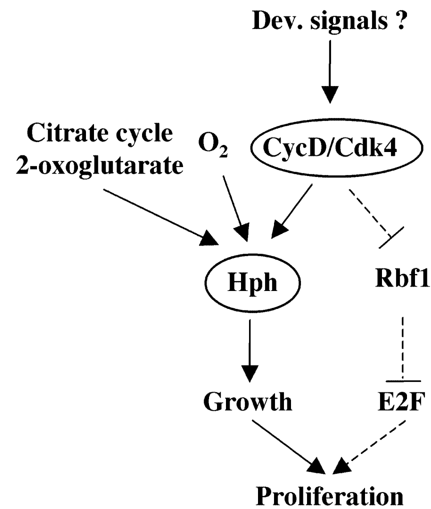

Figure 7. Model for the Interaction of CycD/Cdk4 with Hph

CycD/Cdk4 requires Hph for the induction of cellular growth. CycD/Cdk4 leads to an increase in Hph by regulating its protein levels. Hph is sufficient to drive growth and requires the cofactors oxygen and 2-oxoglutarate for its activity. See text for details.

caused by CycD/Cdk4 or Hph (Figure 5C), Hph's hydroxylation activity is probably required. The only characterized hydroxylation target of Hph is Hif-1α, a mediator of the transcriptional response to hypoxia (Semenza, 2001; Kaelin, 2002; Bruick, 2003). Although mutant alleles of the *Drosophila* Hif-1α ortholog *sima* were not available for our study, a partial loss-of-function allele of the Hif-1β ortholog, *tango*^{1}, was available. To test the potential role of Hif1 in growth control, we used the ey-Flp/FRT method to generate flies in which the eyes were >80% homozygous mutant for *tango*^{1}. If Hph stimulates growth by hydroxylating Hif-1α and targeting it for degradation, then loss of Hif-1 activity might be expected to result in overgrowth phenotypes. Contrary to this expectation, overgrowth was not observed in *tango*^{1}/*tango*^{1} eyes (data not shown). Moreover, GMR-driven expression of CycD/Cdk4 led to the same degree of overgrowth in *tango*^{1}/*tango*^{1} eyes as in wild-type controls (data not shown). Although these observations weigh against an important role for Hif-1 in Hph-driven growth, it is important to note that *tango*^{1} is not a null allele (Emmons et al., 1999) and that Tango is thought to be expressed in excess over its binding partner, Sima. Thus, further analysis using *sima* mutants and overexpression will be required to definitively test whether Hph drives cell growth via a Hif-1-dependent mechanism or through hydroxylation of novel targets. The finding that only one of the three vertebrate Hph orthologs is required for regulation of Hif-1α levels in vivo (Berra et al., 2003) further suggests that additional targets may be important.

There is little data that suggest a growth function for vertebrate HPH. Rat HPH-1/SM-20 was identified first as a gene upregulated by growth factors or serum (Wax et al., 1994). The induction is very fast and peaks at 60 min after stimulation. Remarkably, this induction does not require de novo protein synthesis, as it is not blocked by the translation inhibitor cyclohexamide (Wax et al.,

1994). The effect on growth upon deregulation of mouse Falkor/HPH-3 is controversial: whereas expression of a C-terminal fragment induced cells to grow faster and to a higher density, expression of a wild-type construct had no effect. An antisense oligonucleotide specific for Falkor induced cells to grow faster (Erez et al., 2002). Thus, the function of vertebrate HPH family member in growth control is still ambiguous.

We show that *Drosophila* Hph has at least two functions: response to hypoxia and regulation of growth. How are they linked? In response to hypoxia, Sima/Tango activity is strongly induced in endoreplicative tissues like trachea, gut and fat body, and to a much lesser extent, in imaginal discs (Lavista-Llanos et al., 2002). Although endopreplicative cells lacking Hph are impaired for growth, ectopic overexpression of Hph in these cells did not increase their size (data not shown). In contrast, in imaginal discs, Hph can increase growth when overexpressed. We speculate that in endoreplicative tissues, Hph's main function is to regulate the hypoxic response and, to a minor extent, growth, whereas in imaginal tissues, Hph's main function is to regulate growth. Taken to the environment of wild *Drosophila*, this suggests that hypoxic conditions, which are often found in fermenting fruit, may induce a strong hypoxic response in endoreplicative tissues. Since these tissues are metabolically highly active, this response may be required for the generation of sufficient ATP by the induction of glycolysis. In imaginal discs, cell cycle progression is not controlled primarily by extrinsic factors but by disc intrinsic growth cues (Britton and Edgar, 1998). Therefore, even under hypoxic stress, growth and development of imaginal discs continues but may be slowed down, presumably by inactivation of Hph activity, in order to ensure the formation of adult animals.

In fat body cells, Hph is a nuclear protein, and homozygous Cdk4 mutant cells lack detectable Hph levels (data not shown). Moreover, ectopic expression of CycD/Cdk4 leads to more Hph protein in the cytoplasm and/or the nucleus. Surprisingly, a reporter line showed an increase, rather than a decrease, in Sima activity upon expression of CycD/Cdk4 (data not shown). We propose that in the fat body, Hph induced by CycD/Cdk4 is not sufficient to hydroxylate Hif-1α. In addition to the cofactors oxygen and iron, hydroxylation activity requires the binding of 2-oxoglutarate to the active site of HPH (Epstein et al., 2001; Bruick and McKnight, 2001). 2-oxoglutarate is an intermediate of the citrate cycle, and its levels might correlate with the metabolic activity of the cell. Therefore, Hph protein may be induced by CycD/Cdk4 but may require 2-oxoglutarate and oxygen for catalytic activity in the fat body. In this model, Hph would integrate the regulation of growth by CycD/Cdk4 and its upstream regulators, with the regulation of growth by the metabolic activity, mediated by oxygen and 2-oxoglutarate.

Experimental Procedures

Fly Stocks

UAS-CycD UAS-Cdk4 (Datar et al., 2000); l(3)02255 (BDGP); l(3)S030304 (Szeged Stock Center); Df(3R)6-7, DF(3R)110, Df(3R)3-4, GMR-Gal4, EP(3)3200, en-Gal4, FRT^{82B} Ub-GFP^{27C} (Bloomington); *cdk4*^{3}/CyO-GFP (Meyer et al., 2000); Act>CD2>GAL4 UAS-GFP^{S65T} (Neufeld et al., 1998).

UAS-Hph Transgenes

Full-length cDNA clone GH23732 (ResGen) was digested with XhoI and BglII and ligated into the same restriction sites of pUAST (Brand and Perrimon, 1993). The construct was injected into embryos using standard protocols, and flies carrying P[w^{+} UAS-Hph] on the second or third chromosome were obtained. UAS-HphII.1 and UAS-HphII.2, driven from the heat-shock Gal4 driver in the absence of a heat shock, suppressed the lethality of *hph*^{02255}/*hph*^{S030304}.

Anti-Hph Antiserum

Full-length GST-Hph was created by PCR from GH23732. Primers: 5′-CGCCTCGAGATGATAACCTCCACGACCACGG and 5′-CGCCTCGAGTAGCTGCGCTGTCGGCTTGGG to generate 5′ and 3′ XhoI sites and cloned into the XhoI site of pGEX-4T1 (Pharmacia). The fusion protein was induced in bacteria using IPTG, separated on a 12 $\times$ 12 cm SDS-PAGE, cut out, electroeluted, and concentrated using Amicon columns (Millipore). Rabbits were injected and bled using standard protocols (R&R Research and Development). Serum was affinity purified and eluted at low pH.

Plasmid Rescue

Integration site of PlacW$^{l(3)S030304}$ was determined by plasmid rescue. Total DNA was digested with EcoRI, ligated, transformed into bacteria, and selected for ampicillin resistance. Primers specific for PlacW: sense, 5′-CCGACAAGCTTTGCGTACTCGC; and antisense, 5′-CAGGGTTATTGTCTCATGAGCGG. The PCR product was sequenced and aligned to the genome sequence (BDGP).

Histology

For wing imaginal discs, inverted third instar larvae were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 1 hr and permeabilized in PBS + 1% Triton X-100. Primary antiserum was rabbit α-Hph at 1/40 dilution in PBS + 0.3% Triton X-100. Secondary was Alexa Fluor 568 conjugated goat α-rabbit (Molecular Probes), preabsorbed at 1/1,000 and used at 1/10,000. DNA was stained with DAPI at 0.5 μg/ml. After each staining, carcasses were washed extensively in PBS + 0.3% Triton X-100. Tissues were mounted in Fluoro Guard (Bio Rad). Images were taken on an Olympus Deltavision microscope using a 60$\times$ objective. Z sections of 0.2 μm were deconvolved and three sections were projected. For fat bodies, inverted carcasses were fixed in 8% paraformaldehyde for 1 hr, washed in PBS + 0.1% Triton X-100, and imaged on a Leitz DMRD microscope. Electron microscopy was performed on a JEOL JSM5800 scanning electron microscope.

Northern Blot Analysis

Total RNA was isolated using Trizol reagent (Life Technologies), and the same amount of RNA from the equivalent of three third instar larvae was run on a formaldehyde gel and probed with in vitro Sp6 transcribed pOT-GH23732 digested with BglII. Probing for ribosomal protein S3 verified equal loading.

Clone Size Measurements

Larvae from 4 hr egg collection were transferred to yeasted vials, 50 larvae/vial, 24 hr after egg deposition (AED). Overexpression clones were induced using the hs-Flp Act>CD2>Gal4/UAS system at 37°C for 10 min at 66 hr AED, and third instar larvae were dissected at 114 hr AED. Wing discs were dissected, mounted, imaged on a Leitz DMRD microscope using a 20$\times$ objective, and the size of clones expressing GFP was measured in Photoshop. Imaging and quantitation were done blind, and control was set to one. P values were calculated using standard t test with unequal variances compared to the control in the wild-type background. For FACS, a strong heat shock was applied, and samples were analyzed as in Neufeld et al. (1998).

RT-PCR

Total RNA was isolated using the Trizol isolation reagent (Life Technologies). Reverse transcription was performed using SuperScript (Invitrogen), and the levels of transcripts were analyzed by PCR for *hph*, *tubulin* as a loading control, and GFP as a control for the induction (T. Reis and B.A.E., submitted). Input DNA was at 3-fold serial dilutions. Primers were Hph 5′-CTTTCGGGAACTTTTGGACA

and 5′-CGCAGGAATTACTCGACACA. Tubulin and GFP primers are available upon request. PCR products were run on a 2% agarose gel and stained after the run with SYBR Green I nucleic acid gel stain (Molecular Probes), and the gel was scanned on a Typhoon 8600 scanner. Note: only 1/9 and 1/27 dilutions of ectopic *hph* expression were taken for quantitation. Levels are an underestimation due to saturated levels.

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