

The hypoxia-induced paralogs *Scylla* and *Charybdis* inhibit growth by down-regulating S6K activity upstream of TSC in *Drosophila*

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Diverse extrinsic and intrinsic cues must be integrated within a developing organism to ensure appropriate growth at the cellular and organismal level. In *Drosophila*, the insulin receptor/TOR/S6K signaling network plays a fundamental role in the control of metabolism and cell growth. Here we show that *scylla* and *charybdis*, two homologous genes identified as growth suppressors in an EP (enhancer/promoter) overexpression screen, act as negative regulators of growth. The simultaneous loss of both genes generates flies that are more susceptible to reduced oxygen concentrations (hypoxia) and that show mild overgrowth phenotypes. Conversely, *scylla* or *charybdis* overactivation reduces growth. Growth inhibition is associated with a reduction in S6K but not PKB/Akt activity. Together, genetic and biochemical analysis places *Scylla/Charybdis* downstream of PKB and upstream of TSC. Furthermore, we show that *scylla* and *charybdis* are induced under hypoxic conditions and that *scylla* is a target of *Drosophila* HIF-1 (hypoxia-inducible factor-1) like its mammalian counterpart RTP801/REDD1, thus establishing a potential cross-talk between growth and oxygen sensing.

[*Keywords:* Insulin signaling; S6 kinase; TSC; hypoxia; RTP801; REDD1]

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The control of growth in response to intrinsic and extrinsic factors is important for the development of every organism. Deregulated growth can have devastating consequences such as tumor formation. The evolutionarily conserved Insulin/IGF receptor (Inr)/Target of Rapamycin (TOR) signaling network plays an important role in modulating growth, metabolism, reproduction, and life span in response to intracellular and extracellular signals in species ranging from invertebrates to humans (Garofalo 2002; Jacinto and Hall 2003). In *Drosophila*, viable mutant combinations of positive components of the *Drosophila* Inr cascade such as *Inr*, *chico* (the homolog of vertebrate *IRS1-4*), *PKB* (*Protein kinase B*, also known as *Akt*) and *PDK1* (*3-phosphoinositide-dependent protein kinase-1*) lead to developmentally delayed and proportionally reduced small flies, displaying a reduction in cell size and number (Bohni et al. 1999; Brogiolo et al. 2001; Rintelen et al. 2001). On the other hand, loss of PTEN (phosphatase and tensin homolog on chromosome ten), which antagonizes PI3K activity by dephosphory-

lating the 3'-position of phosphoinositides, leads to hypertrophy and hyperplasia (Goberdhan et al. 1999; Huang et al. 1999; Gao et al. 2000). In humans, loss of the tumor suppressor PTEN is observed frequently in glioblastomas, prostate cancers, and endometrial cancers, and *PTEN* germline mutations are linked to dominant hamartoma syndromes like Cowden syndrome, Lhermitte-Duclose disease, Proteus syndrome, and Bannayan-Zonana syndrome (Sulis and Parsons 2003). Genetic studies in *Drosophila* indicate that PKB has a crucial role in signaling downstream of PTEN since flies completely lacking PTEN function can be rescued to viability by lowering PKB activity (Stocker et al. 2002).

The TOR/S6K (S6 kinase) branch of the growth modulatory network is negatively regulated by the tumor suppressor Tsc1 (hamartin)/Tsc2 (tuberin) complex. Tuberous sclerosis complex (TSC) is an autosomal-dominant disorder characterized by the formation of hamartomas, benign tumors that arise in various tissues (Pan et al. 2004). In *Drosophila*, cells devoid of Tsc1/Tsc2 function are increased in size (Gao and Pan 2001; Potter et al. 2001; Tapon et al. 2001). Tsc2 and, more weakly, Tsc1 were found to physically associate with dTOR, thereby inhibiting dTOR kinase activity (Gao et al. 2002). Other

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studies reported an inhibitory role of PKB on Tsc1–Tsc2 by PKB-mediated phosphorylation of Tsc2 (Inoki et al. 2002; Manning et al. 2002). Recently, the small GTPase Rheb (Ras homolog enriched in brain) has been identified as a new positive growth effector acting downstream of Tsc1–Tsc2 and upstream of TOR. Mechanistically, Tsc2 acts as GTPase-activating protein (GAP) toward Rheb (Garami et al. 2003; Inoki et al. 2003a; Saucedo et al. 2003; Stocker et al. 2003; Tee et al. 2003; Zhang et al. 2003). The molecular mechanism how Rheb relays the signal to TOR is currently unknown. *dTOR* mutants show a growth deficit that is more pronounced in endoreplicative tissues than in mitotic tissues (Oldham et al. 2000; Zhang et al. 2000). An effector of mTOR is S6K, which upon activation by mTOR phosphorylates ribosomal protein S6. S6K-mediated S6 phosphorylation has been thought to lead to a preferential translation of mRNAs encoding ribosomal proteins and proteins of the translational apparatus although the significance of this S6K function has been questioned recently (Tang et al. 2001; Stolovich et al. 2002; Pende et al. 2004). Inr/TOR signaling activity culminates in the regulation of translation rate by controlling S6K and the translational repressor 4E-BP1 (Gingras et al. 2004). *S6K* mutant flies are small but in contrast to mutants of the Inr pathway, only cell size is reduced without a change in cell number (Montagne et al. 1999). Therefore, loss of S6K function reduces growth and body size to a lesser extent than loss of other positive components acting further upstream in the cascade.

Growth is modulated by extrinsic factors such as nutrients, temperature, and hypoxia (Palos and Blasko 1979; Frazier et al. 2001; Azevedo et al. 2002). However, their link to the Inr/TOR signaling network are not well defined. Although it is known that starvation results in a reduction in the levels of insulin-like peptides and a reduction in S6K activity in *Drosophila* (Oldham et al. 2000; Ikeya et al. 2002), little is known about whether temperature or hypoxia regulates the activity of this pathway. It is conceivable that mutations in genes coding for factors mediating the modulation of growth in response to external stimuli have escaped detection because they do not exhibit a very strong phenotype under standard culture conditions. For example, overexpression of the Forkhead transcription factor FOXO3a, the human homolog of *Drosophila* dFOXO, produces a very subtle small eye phenotype under normal nutrient conditions. Under starvation, however, this phenotype is massively exacerbated, inducing a further eye size reduction and cell death. Furthermore, *dFOXO* mutant flies are viable and do not show an (over)growth phenotype in an otherwise wild-type background under normal conditions (Junger et al. 2003).

Genes like *dFOXO* were missed in genetic loss-of-function screens aimed at identifying growth-regulatory genes (1) because they have only mild or missing mutant phenotypes under normal conditions and/or (2) because their function is masked by redundancy.

Genes acting in a redundant manner can be identified in a complementary gain-of-function approach using EP

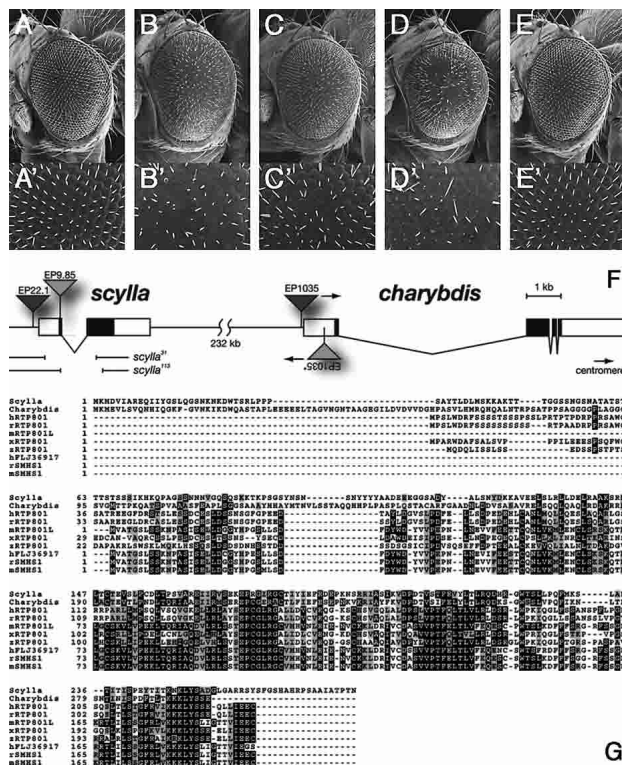
(enhancer/promoter) elements (Rorth 1996). Screening >4000 novel EP lines, we found two insertions in the *scylla* locus as suppressors of a PKB/PDK1-dependent eye overgrowth phenotype. We identified a second gene in the *Drosophila* genome with homology to *scylla* named *charybdis*. Homologs of these genes also exist in mammals, and they have been implicated in the response of a cell to hypoxia or more generally as stress-induced genes having either pro- or anti-apoptotic functions. We present evidence that *scylla* and *charybdis*, like some of their mammalian homologs, are induced by hypoxia and that Scylla and Charybdis act as partially redundant negative regulators of growth by controlling S6K but not PKB activity.

Results

Identification of *scylla* and *charybdis*

In order to identify novel genes involved in growth regulation by the Inr/TOR pathway, we performed an EP overexpression screen using a double-headed EP element. We used a genetically sensitized system involving coexpression of PKB and *PDK1* (achieved by using *EP837* that drives endogenous *PDK1*) in the eye, which leads to a big eye phenotype (Fig. 1B). Pilot experiments demonstrated that overexpression of *PTEN* or a dominant-negative version of the catalytic subunit of *Drosophila* PI3K, Dp110, were not able to suppress the PKB/PDK1-dependent phenotype. Thus, our screening system is likely to identify components acting downstream of or in parallel to PKB/PDK1. For example, coexpression of *Tsc1/Tsc2* strongly suppresses the phenotype of our tester flies (data not shown).

We identified two EP insertions (*EP22.1*, hereafter named *EPscyl*, and *EP9.85*) in the *scylla* locus as suppressors of the PKB/PDK1 bulging eye phenotype (Fig. 1C). BLASTP search with the Scylla amino acid sequence revealed another homologous protein encoded in the *Drosophila* genome termed Charybdis. *scylla* (*scy*, CG7590) and *charybdis* (*char*, CG7533) are separated by ~232 kb of genomic DNA. Their gene products share a high degree of homology (38% identity, 49% similarity), suggestive of a gene duplication event (Fig. 1F,G). We checked whether *charybdis* overexpression would behave similarly to *scylla* in the PKB/PDK1 overexpression assay using *EP1035* (hereafter named *EPchar*). Indeed, the big eye phenotype of the tester system is also suppressed by *EPchar* (Fig. 1D). UAS transgenes with either the *scylla* or *charybdis* cDNA recapitulate the suppression phenotype of the corresponding EP element (data not shown). Coexpression of *scylla* and *charybdis* further ameliorates the suppression phenotype to a nearly wild-type situation (Fig. 1E). Notably, *scylla* or *charybdis* overexpression on their own using a panel of different eye/wing Gal4 drivers reduced adult organ size. Coexpression of the caspase inhibitors p35 or DIAP1 did not rescue the small eye phenotype induced by expression of either *scylla* or *charybdis* in the eye. Moreover, no elevated cell death in eye imaginal disks overexpressing *scylla*/



charybdis under control of the *GMR-Gal4* driver was observed by acridine orange staining (data not shown). This suggests that apoptosis is not the cause for the eye size reduction. Thus, *scylla* and *charybdis* overexpression antagonizes the growth-promoting effects of PKB/PDK1 and is sufficient to negatively regulate growth.

scylla and *charybdis* are both expressed during embryogenesis in dynamic, partially overlapping patterns. In contrast to the broadly expressed *scylla* mRNA, *charybdis* transcripts are predominantly restricted to neurons of the CNS and PNS as assessed by mRNA in situ hybridization. During late larval stages *scylla* mRNA is uniformly expressed without apparent tissue-specific distribution, whereas *charybdis* mRNA expression could not be detected in third instar imaginal disks (data not shown).

Scylla and *Charybdis* belong to an evolutionarily conserved protein family

scylla codes for a 280-amino acid polypeptide with a predicted molecular weight (MW) of ~31 kDa, and the *Charybdis* protein contains 299 amino acid residues (MW ~32 kDa). Since *EP9.85* is located in the coding region of *scylla*, thus generating a Scylla protein with an N-terminal truncation of 12 amino acids, we tested whether a *UAS-scly* transgene encoding the truncated form of Scylla behaves as the full-length protein. Overexpression of *UAS-scly*^{Δ1–12} and *UAS-scly*^{wt} showed the same effects as *EPscly* (data not shown), indicating that amino acids 1–12 are dispensable for the growth-suppressing function of Scylla.

Homologs of Scylla/Charybdis exist throughout the animal kingdom including humans, rat, mouse, *Xenopus*, and zebrafish. Reminiscent of the situation in *Drosophila*, mammals possess several paralogous proteins with homology to Scylla/Charybdis. At least two homologs (RTP801/REDD1 and FLJ3691, a hypothetical protein presumably corresponding to REDD2 [Ellisen et al. 2002]) exist in humans, two (RTP801 and SMHS1) in rat, and three (RTP801L, Dig2, and SMHS1) in mice. The overall homology is highest toward the C terminus (Fig. 1G). Charybdis contains a predicted coiled-coil structure (amino acids 168–188).

In mammals, *RTP801/REDD1* and other members of the family are induced upon various forms of cellular stress including hypoxia, DNA damage, and dexamethasone treatment (Ellisen et al. 2002; Shoshani et al. 2002; Wang et al. 2003). In fact, *RTP801/REDD1* is a direct target of HIF-1, a heterodimeric transcription factor that plays a pivotal role for survival in response to hypoxia (Shoshani et al. 2002). Furthermore, *RTP801/REDD1* is controlled by p53 and p63 [Ellisen et al. 2002]. Depending on the experimental setup and cell context, *RTP801* overexpression either inhibits or increases apoptosis, suggesting a complex regulation and/or dependence on the developmental program of the cell (Shoshani et al. 2002).

Scylla and *Charybdis* inhibit growth and are important under hypoxic conditions

To investigate the function of Scylla and Charybdis in more detail, we generated loss-of-function mutations in both genes and complemented the analysis with overexpression studies.

Partial *scylla* deletions were obtained by imprecise excisions of *EP9.85*, which is integrated in the *scylla* open reading frame (ORF) and therefore already represents a *scylla* allele (hereafter named *scly*^{EP9.85}) (Fig. 1F). For *charybdis*, we used a local hop strategy of *EPchar* to obtain *char*¹⁸⁰, constituting a new EP insertion (*EP1035**) in the *charybdis* 5'-untranslated region (UTR) plus the original *EPchar* (see Materials and Methods). Quantitative real time-PCR showed that in *char*¹⁸⁰ homozygotes, *charybdis* mRNA expression is decreased to ~25% of wild-type levels (data not shown). We assume

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that this *charybdis* allele is a strong hypomorph, as mRNA levels were only slightly more reduced (to 23% or 15%, respectively) when RNA was extracted from flies heterozygous for *char¹⁸⁰* over either one of two independent deficiencies uncovering *charybdis* and *scylla* (data not shown).

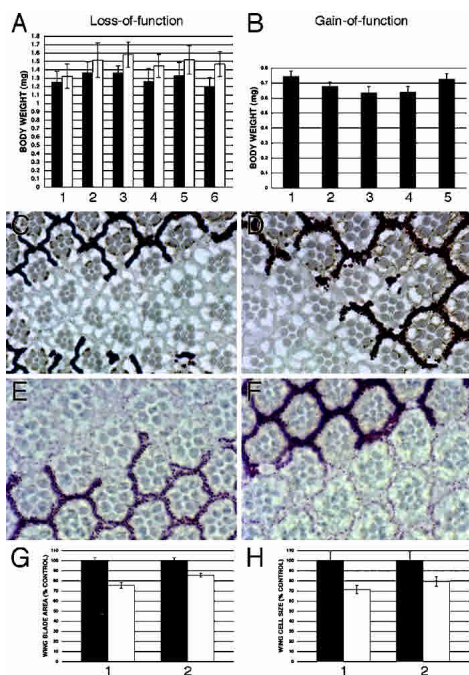
All *scylla* mutant combinations and the *char¹⁸⁰* homozygotes are viable and fertile without apparent mutant phenotype. *scylla* and *char¹⁸⁰* mutant animals have the same weight as control flies. Measurement of wing size and hair density in the adult wing of *scylla* mutants revealed no differences in cell size and cell number as compared to control animals (data not shown). We created *scylla* loss-of-function clones in imaginal disks using FLP/FRT-mediated mitotic recombination to test the effect on growth properties of the mutant tissue. One would expect a growth advantage of cells in clones lacking a bona fide negative growth regulator, as is the case for *PTEN*. However, larval *scylla* mutant clones were the same size as their wild-type sister clones. Likewise, clones obtained in adult eyes revealed no increase in cell size of *scylla* or *char¹⁸⁰* mutant ommatidia (data not shown). Thus, loss of Scylla or Charybdis function is dispensable for growth under normal conditions. It is conceivable that Scylla and Charybdis act in a redundant manner. Therefore, it was important to create *scylla charybdis* double mutants.

We combined *char¹⁸⁰* with three of our *scylla* alleles by meiotic recombination. All double-mutant *scylla charybdis* combinations produced viable adult flies. Weight analysis of heteroallelic *scylla charybdis* flies demonstrated that simultaneous loss of Scylla and Charybdis significantly increases body weight. Consistent results were obtained by combining one copy of *scy^{EP9.85/31/113} char¹⁸⁰* and the deficiency *Df(3L)vin4*

uncovering *scylla* and *charybdis*. Mutant females were on average 6%–23% and males 9%–17% heavier than control flies (Fig. 2A; data not shown). Conversely, ubiquitous *scylla/charybdis* overexpression using the *Act5CGal4* driver generated flies that are decreased in size and weight (15.1% using *EPscyl* and 14.3% using *UAS-char#53*) (Fig. 2B, data only shown for males). The majority of *scylla/charybdis*-overexpressing flies eclosed with a minor delay (0.5–1 d) (data not shown).

To assess whether *charybdis/scylla* overexpression affects cell size, we generated *scylla* and *charybdis* gain-of-function flip-out clones in the eye, marked by the absence of the red pigment. A moderate reduction in cell size was observed in cells overexpressing *scylla* or *charybdis* (Fig. 2C,D). Ommatidia overexpressing *scylla*

Figure 2. Scylla and Charybdis inhibit growth. (A) Simultaneous loss of Scylla and Charybdis leads to an increase in body weight. Control flies (black bars) are represented by either of the two possible double mutants over the *TM3* balancer chromosome (first three black columns) or the deficiency chromosome (*Df(3L)vin4*) over *TM3* (three last black columns) and were derived from the same vial as the heteroallelic double-mutant flies, $p < 0.009$ using Student's *t*-test (two-tailed). Between 32 and 65 flies were weighed per genotype. Genotypes (only white bars indicated) are as follows: *y w; scy¹¹³ char¹⁸⁰/scy^{EP9.85} char¹⁸⁰* (1); *y w; scy³¹ char¹⁸⁰/scy^{EP9.85} char¹⁸⁰* (2); *y w; scy³¹ char¹⁸⁰/scy¹¹³ char¹⁸⁰* (3); *y w/+; scy^{EP9.85} char¹⁸⁰/Df(3L)vin4* (4); *y w/+; scy¹¹³ char¹⁸⁰/Df(3L)vin4* (5); *y w/+; scy³¹ char¹⁸⁰/Df(3L)vin4* (6). (B) Ubiquitous *scylla* and *charybdis* overexpression generates smaller flies that have reduced body weights. In all our overexpression studies, we obtained the strongest effects using *EPscyl* or *UAS-char#53*, driving *scylla* and *charybdis*, respectively. A representative experiment of three independent weighing analyses is shown ($n = 35$ for each genotype; $p < 0.0001$ for *UAS-scy#2*, *EPscyl*, *UAS-char#53*, and $p < 0.02$ for *EPchar*). Data are only shown for males, but significant weight reduction is also observed in females. Genotypes are as follows: *y w; Act5CGal4/+* (control) (1); *y w; UAS-scy#2/+; Act5CGal4/+* (2); *y w; Act5CGal4/EPscyl* (3); *y w; Act5CGal4/UAS-char#53* (4); *y wAct5CGal4/EPchar* (5). (C–F) Tangential sections through the adult eye showing photoreceptor and associated ommatidial cells. Clones of cells either overexpressing *scylla/charybdis* or lacking the function of both are recognizable by the absence of red pigment. *scylla*-overexpressing cells (C) or *charybdis*-overexpressing cells (D) in the eye are smaller than neighboring wild-type cells, and no patterning defects are observed. Conversely, *scylla charybdis* double-mutant cells show a mild increase in cell size (E,F). Genotypes are as follows: *y w hsf1p; GMR > w⁺ > Gal4/EPscyl* (C); *y w hsf1p; UAS-char#56/+; GMR > w⁺ > Gal4/+* (D); *y w hsf1p; FRT80 scy¹¹³ char¹⁸⁰/FRT80 w⁺* (E); *y w hsf1p; FRT80 scy^{EP9.85} char¹⁸⁰/FRT80 w⁺* (F). (G,H) Quantification of wing cell size and cell number of flies overexpressing *scylla*. Hair density in a defined square area next to the junction of the posterior cross-vein and vein five was determined, and from this number cell size was calculated. The cell number corresponds to the ratio of total wing area and cell size. *scylla* overexpression reduces only cell size but not cell number. Black bars always represent the control (*y w; Act5CGal4/+*), and white bars represent *scylla* overexpression (*y w; Act5CGal4/EPscyl*). Numbers indicate the sex: 1 for males, and 2 for females. We analyzed 10 wings of each genotype.



or *charybdis* exhibit no patterning defects. On the other hand, simultaneous removal of Scylla and Charybdis in clones of photoreceptor cells resulted in slightly enlarged cells (Fig. 2E,F). Consistently, when most of the head capsule and the eyes were made homozygous by means of the eyflp/FRT system in an otherwise heterozygous mutant background, a mild big head phenotype was generated in the double mutant but not in either single mutant (Supplementary Fig. S1).

The gain-of-function clonal analysis in the eye showed that cell size is reduced upon forced *scylla/charybdis* expression, but it did not address the question whether cell number is affected. To clarify this issue, the wing size and cell number of flies that ubiquitously overexpress *scylla* were measured. The insect wing is a double-layered epithelial structure, and each cell in the wing secretes a single hair (trichome). Therefore, by counting the number of trichomes per defined area, hair density can be taken as a measure for cell number. Overall wing size was reduced by ~25% in males or ~15% in females overexpressing *scylla* (Fig. 2G). By extrapolating the number of cells per measured area, we found that cell size is decreased by ~29% in males and ~21% in females (Fig. 2H). We conclude that the size reduction brought about by overexpressing *scylla* is caused by a reduction in cell size. Cell density is even slightly increased (5.8% in males, 7.6% in females) (data not shown). Our data show that Scylla and Charybdis have a growth-inhibitory role and that they share some functional redundancy.

RTP801/REDD1 was shown to be induced by hypoxia (Shoshani et al. 2002). This prompted us to investigate the effects of hypoxia on the *scylla charybdis* double mutants. *scylla charybdis* mutant larvae were raised on normal food at room temperature in a hypoxia chamber containing 9% oxygen during their entire development. In general, control flies as well as *scylla charybdis* homozygotes were 3–4 d delayed in development under these hypoxic conditions. However, whereas adult homozygous *scylla charybdis* double mutants could readily be recovered under standard culture conditions in normoxia, homozygous mutants of three independent *scylla charybdis* allelic combinations (*char*¹⁸⁰ in combination with *scy*³¹, *scy*¹¹³, or *scy*^{EP9,85}) were strongly underrepresented compared to normoxic conditions. We also observed the appearance of an increased number of dead pupae in the vials (data not shown). The *scylla*¹¹³ *char*¹⁸⁰ double-mutant combination had the strongest effect, and only two escapers (out of 326 scored flies) hatched, whereas under normoxia flies with this genotype were recovered with nearly the expected Mendelian ratio. The eclosed homozygotes raised under hypoxia did not show obvious morphological defects.

Thus, whereas simultaneous loss of Scylla and Charybdis under normoxic conditions results in a slight increase in growth, their absence under reduced oxygen concentrations severely compromises larval development. This indicates that Scylla and Charybdis have a critical function for survival under hypoxic conditions.

scylla and charybdis are up-regulated under hypoxic conditions and scylla is a target of Drosophila HIF-1

The transcription factor HIF-1 is the key regulator of changes in gene expression in response to hypoxia (Bruick 2003). It consists of two bHLH-PAS domain protein subunits (HIF-1 α and HIF-1 β). Under conditions of low oxygen, the HIF-1 protein complex is stabilized and binds to Hypoxia Response Elements (HRE), short regulatory DNA sequences (core recognition sequence 5'-TACGTG-3') located in the genomic region of target genes. Both the *scylla* and *charybdis* loci possess several HREs. Since *RTP801/REDD1*, a mammalian homolog of *scylla* and *charybdis*, is a direct target gene of HIF-1 and is induced under hypoxic conditions, we wondered whether this function is evolutionarily conserved. We subjected wild-type larvae to hypoxia (between 2% and 5% O₂) and checked for the induction of *scylla* and *charybdis* expression. It is mainly the endoreplicative tissue such as fat body, gut, salivary glands, and tracheae that respond to changes in oxygen concentrations (Lavista-Llanos et al. 2002). *scylla* mRNA expression was up-regulated in the larval fat body and in the gut after hypoxia (Fig. 3B; data not shown). *charybdis*, on the other hand, was mildly induced in the midgut but not in the fat body (Fig. 3D; data not shown). We also tested whether hypoxia had an effect on Scylla protein levels and distribution. To detect the endogenous Scylla protein, we took advantage of a transgenic Scylla-reporter line (a so-called protein trap line). This protein trap line bears a promoter-less green fluorescent protein (GFP)-reporter transgene in the *scylla* locus generating a Scylla-GFP fusion protein (Morin et al. 2001). Scylla-GFP is expressed in most larval tissues. Under normoxic conditions, nuclear accumulation of Scylla protein is observed in some cells of the endoreplicative tissue (data not shown). Consistent with our mRNA data, upon exposure of third instar larvae to various hypoxia conditions, we observed an up-regulation and nuclear localization of Scylla protein in the fat body (data not shown) and in the gut (Fig. 3K–P).

In *Drosophila*, the bHLH-PAS family comprises Period (Per), Trachealess (Trh), Single-minded (Sim), Spineless (Ss), Dysfusion (Dys), Tango (Tgo), and Similar (Sima). Tgo is the ubiquitously expressed HIF-1 β ortholog, which dimerizes with any of the α -subunits. Sima has been shown to fulfill analogous functions to its mammalian homolog HIF-1 α (Lavista-Llanos et al. 2002).

To test whether *scylla/charybdis* transcription is regulated by bHLH-PAS proteins that recognize the same DNA stretch, we overexpressed *sim*, *trh*, or *sima* together with *tgo* using the *Lsp2Gal4*-driver that is active specifically in the fat body during the third larval stage. For Sima, we used a form lacking the oxygen-dependent degradation domain (ODD), rendering it refractory to proteolytic destruction under normoxic conditions. Only the coexpression of *tgo* and *sima* induced *scylla* but not *charybdis* expression as assessed by mRNA in situ hybridization (Fig. 3G,*j*; data not shown). This does not preclude, however, the possibility that *charybdis* is a

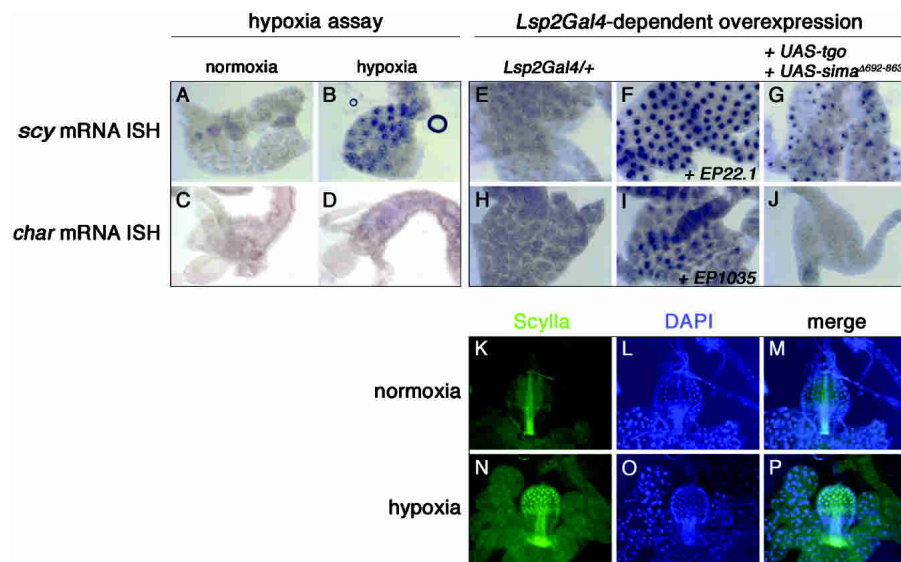


Figure 3. *scylla* and *charybdis* are induced by hypoxia, and *scylla* is a target of *Drosophila* HIF-1. (A–D) Larvae were incubated in a hypoxia chamber for either 17 h under 2% O₂ or for 7 h under 2.8% O₂ conditions leading to the induction of *scylla* (B) and *charybdis* (D) expression as assessed by mRNA in situ hybridization (ISH). Since *scylla* mRNA is detected in the gut under normoxia, it has been difficult to assess whether *scylla* transcript levels are further induced under hypoxia (data not shown). (E–J) Directed coexpression of Tgo and Sima in the fat body up-regulates *scylla* (G) but not *charybdis* (J) transcription. (F, I) Positive controls for *scylla* and *charybdis*, respectively. (K–P) Scylla protein localizes to the nucleus and is up-regulated in the proventriculus (N) and the fat body (not shown) in hypoxia. Genotypes are as follows: *y w* (A–D); *y w; Lsp2Gal4/+* (E, H); *y w; Lsp2Gal4/EPscyl* (F); *y w; UAS-tgo/+; Lsp2Gal4/UAS-sima^{Δ692–863}* (G, J); *y w; Lsp2Gal4/EPchar* (I). (K–P) Scylla fly trap larvae grown in normoxia (K–M) or incubated in hypoxia (2.8% O₂ for 8 h; N–P).

target of Tgo–Sima as we detected its endogenous induction upon hypoxia only in the gut but not in the fat body. Since neither expression of *trh* with *tgo* nor *sim* induced *scylla* or *charybdis* expression, the regulation of *scylla* by the Tgo–Sima heterodimer is specific. Thus, *scylla* and *charybdis*, like their mammalian homolog *RTP801/REDD1*, are induced by hypoxia, and at least *scylla* appears to be a direct target gene of the HIF-1 homolog Tgo–Sima.

Scylla acts downstream of PKB but upstream of TSC

Although loss of Scylla function does not produce a mutant phenotype on its own, we tested whether it would alter the PKB/PDK1 overexpression eye phenotype. Indeed, loss of Scylla function enhanced the PKB/PDK1 overgrowth phenotype (Fig. 4B,C). Thus, Scylla is essential for attenuating the increased growth in response to hyperactivation of the Inr pathway. Furthermore, loss of Scylla partially suppressed the growth reduction associated with reduced PKB function as assessed by comparing weights of PKB³ single mutants to *scy*³¹ PKB³ double mutants (Fig. 4D). In contrast, complete loss of Scylla in a heteroallelic *S6K* combination did not rescue the *S6K* single mutant phenotype indicating that *S6K* is epistatic over *scylla* (Fig. 4E).

Moreover, overexpression of *scylla* and *charybdis* not only suppressed the growth phenotype caused by overactivation of the Inr pathway in the eye but to a certain

extent also rescued the lethality associated with the ubiquitous increase in Inr pathway activity due to either overexpression of PKB or loss of PTEN. *scylla* rescued the male-specific lethality caused by ubiquitous expression of PKB and organismal lethality associated with the partial but not complete loss of PTEN function. Similarly, PKB-associated male lethality was also rescued by *charybdis* overexpression (Supplementary Tables 1, 2). This indicates that *scylla* and *charybdis* have the capacity to act as potent negative regulators of insulin signaling downstream of PKB and PDK1.

Several lines of evidence suggest that Scylla and Charybdis act upstream of TSC and Rheb. *Tsc1/2* mutant flies can be rescued to adulthood by reducing S6K signaling (Radimerski et al. 2002a), and a mere reduction of one TOR copy in a *Tsc1* mutant context results in a rescue to the pupal stage (Gao et al. 2002). We examined whether ubiquitous *scylla* overexpression could rescue the larval lethality of heteroallelic *Tsc1/2* mutant combinations (*Tsc1^{2G3}/Tsc1^{Q87X}* and *Tsc2⁵⁶/Tsc2¹⁹²*) using the *daGal4* or *Act5CGal4* drivers in combination with a *UAS-scy* transgene or *EPscyl* at 18°C, 25°C, and 29°C. Ubiquitous overexpression of *scylla/charybdis* in a *Tsc1/2* mutant background did in no case extend larval development beyond first/second instar, and these larvae died at the same time as *Tsc1/2* mutants. Moreover, the big head phenotype of *Tsc2¹⁹²* (and *Tsc2⁵⁶*) induced by the *eyflp/FRT* system was not further enhanced in *scy^{EP9.85} char¹⁸⁰ Tsc2* triple-mutant heads (Fig. 4F,G). We have shown before (Supplementary Fig. S1) that

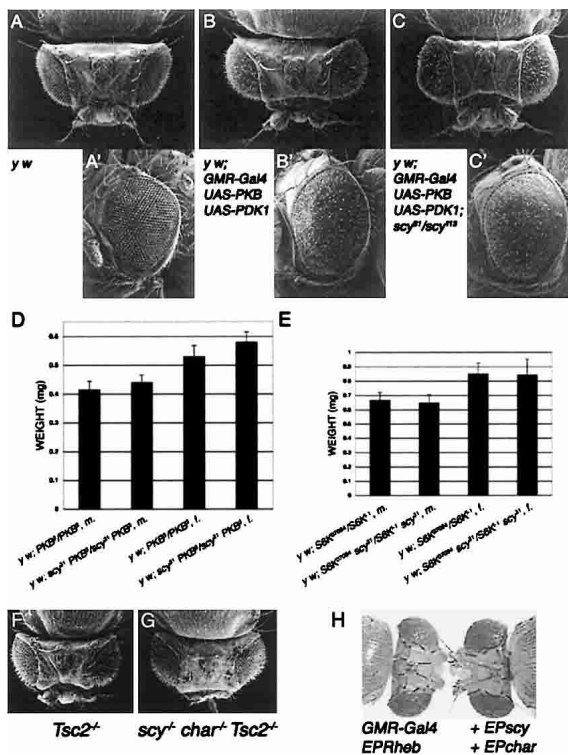


Figure 4. Scylla attenuates growth in response to elevated Inr pathway activity downstream of PKB but upstream of TSC. (A–C) A PKB/PDK1-overexpression eye phenotype is enhanced in a *scylla* mutant background. Genotypes are as follows: *y w* (A,A'); *y w; GMR-Gal4 UAS-PKB UAS-PDK1/+* (B,B'); *y w; GMR-Gal4 UAS-PKB UAS-PDK1/+; scylla³¹/scylla¹¹³* (C,C'). (D) Loss of *scylla* partially suppresses the weight loss of homozygous *PKB³* mutants. Weight increase in the double mutants versus *PKB³* single mutants is ~6% (males) and 10% (females) for this experiment. One representative of four independent weighing experiments is shown; $p < 0.0001$, $n = 60$ (males) and 90 (females). (E) No difference in body weight between a hypomorphic *S6K* mutant combination and *S6K scylla* double mutants was observed ($n = 30$). (F,G) *scylla charybdis Tsc2* triple-mutant heads resemble *Tsc2* single mutants and do not show additive growth effects, indicating that Scylla and Charybdis act upstream of Tsc2. Genotypes are as follows: *y w eyf1p; FRT80 gig¹⁹²/FRT80 c13L w⁺* (F); *y w eyf1p; FRT80 scy^{EP9.85} char¹⁸⁰ gig¹⁹²/FRT80 c13L w⁺* (G). (H) A Rheb-dependent bulging eye phenotype cannot be suppressed by *scylla/charybdis* coexpression. Genotypes are as follows: *y w; GMR-Gal4/+; EPRheb/+* (left); *y w; GMR-Gal4/+; EPRheb/EPscry EPchar* (right).

heads composed almost entirely of *scylla charybdis* double-mutant cells are enlarged. Conversely, *GMR-Gal4*-driven co-overexpression of *Tsc1*, *Tsc2*, and *scylla* or *charybdis* in the eye did not further reduce the small eye phenotype induced by coexpression of *Tsc1* and *Tsc2* on their own (data not shown). The absence of an additive growth effect upon loss of *Tsc2*, *scylla*, and *charybdis* or overexpression of *Tsc1/2* and *scylla* or *charybdis* suggests that they function in the same pathway. These results are consistent with the idea that Scylla and Charybdis act upstream of the TSC complex (see also

next section). Our conclusion is further supported by the fact that neither a Rheb-dependent bulging eye phenotype nor organismal lethality could be suppressed by *scylla/charybdis* coexpression (Fig. 4H; data not shown).

Scylla decreases S6K but not PKB activity

To test whether the placement of Scylla between PKB and TSC can be corroborated biochemically, we investigated the effect of *scylla* overexpression on PKB and S6K activity.

We tested PKB activity of adult female heads overexpressing *scylla* or *charybdis* in conjunction with *PKB* and *PDK1* under control of the *GMR-Gal4* enhancer. The same experimental setup has previously been used to demonstrate that PDK1 increases PKB activity (Cho et al. 2001). Total fly head protein was extracted and PKB activity was assayed by incorporation of ³²P-labeled phosphate into a synthetic PKB substrate (Crosstide, CT). Although *scylla/charybdis* overexpression substantially suppressed the PKB/PDK1-induced bulging eye phenotype (Fig. 1C), PKB activity was not reduced in these eyes (Fig. 5A). Moreover, PKB activity was also unaffected in a *scylla*^{-/-} background (Fig. 5B).

These results are consistent with the placement of Scylla downstream of PKB. To test the effect of Scylla on

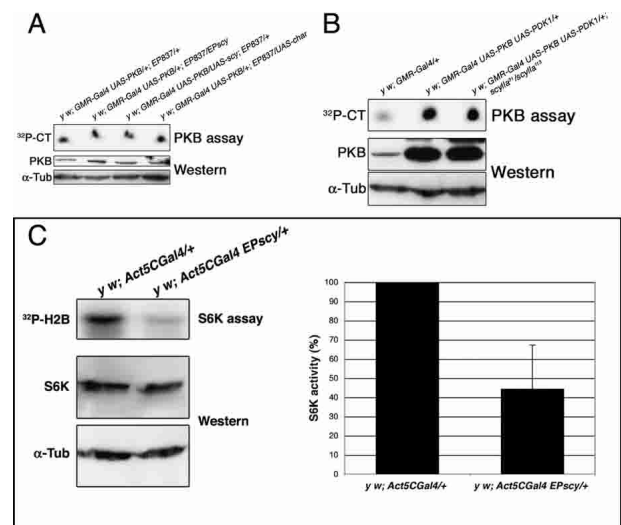


Figure 5. Scylla reduces S6K activity, but neither Scylla nor Charybdis down-regulates PKB activity. (A) Head extracts of flies overexpressing *PKB* and *PDK1* in conjunction with either *scylla* or *charybdis* in the eye were assayed for PKB activity. No reduction could be detected (experiment has been performed twice). Genotypes are indicated above the autoradiograph. (CT) Crosstide, a synthetic PKB substrate. (B) Eye-specific *PKB/PDK1* coexpression in a *scylla* mutant background does not lead to elevated PKB activity (three experiments have been done). (C) Ubiquitous *scylla* overexpression decreases S6K activity by 56% on average, $p < 0.006$. S6K activity of second instar larvae was determined by the incorporation of radioactively labeled phosphate into the H2B substrate. Control (*Act5CGal4/+*) activity was set to 100%. Five independent experiments were performed.

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S6K activity, second instar larvae expressing *scylla* under the control of *Act5CGal4* were collected, and larval extracts were assayed for S6K activity. On average, S6K activity was down-regulated by >50% (Fig. 5C). Although there may also be a slight reduction in total S6K protein levels, this effect cannot account for the much stronger reduction in S6K activity. Taken together with the genetic evidence presented above, these results strongly support the argument that Scylla acts between PKB and TSC to regulate S6K activity. Furthermore, in an accompanying paper, Brugarolas et al. (2004) provide direct biochemical evidence that a functional TSC complex is required for RTP801/REDD1 to affect S6 phosphorylation. Altogether, these data indicate that Scylla functions upstream of TSC.

Scylla and Charybdis prolong life span under starvation conditions

Zinke et al. (2002) have performed a whole-genome DNA microarray analysis of 2-d-old larvae (48 h AEL) grown on normal food that were subsequently subjected to a starvation regime for different time periods. Under these conditions, upon 12 h of starvation, *scylla* and *charybdis* expression were found to be on average 6.3 times and 4.6 times up-regulated, respectively (downloadable Supplemental Material at <http://www.fzk.de/embo>). Therefore, we tested the effects of starvation on the viability of *scylla* and *charybdis* mutants, as well as on flies overexpressing both genes by exposing adult flies to a water-only diet. Various *scylla* heteroallelic combinations did not show elevated susceptibility to starvation (data not shown). However, *char¹⁸⁰* mutants lived significantly shorter lives than control flies, suggesting that Charybdis has a protective effect for the animal under nutrient-deprived conditions (Fig. 6A). Strikingly, forced expression of *scylla/charybdis* extended mean life span by up to twofold (Fig. 6B). We analyzed lipid and glycogen content of these flies to see whether energy stores were altered. Indeed, flies overexpressing *scylla* and/or *charybdis* showed significantly elevated lipid levels (Fig. 6C). We also measured glycogen levels but could not detect any statistically significant changes, although there was a tendency toward increased glycogen content (data not shown).

Discussion

Here we provide evidence that two related proteins, Scylla and Charybdis, are negative modulators of Inr/TOR signaling in response to different external stress situations including hypoxia and starvation. *scylla* and *charybdis* single mutants do not show obvious growth phenotypes. *scylla charybdis* double-mutant flies are also viable and fertile and exhibit a slight increase in body size. Viability of the double mutants is strongly reduced, however, when they are reared under hypoxic conditions. Thus, although Scylla and Charybdis are largely dispensable for normal development, they have a critical role for the endurance of prolonged hypoxia. We show that *scylla* is transcriptionally induced as a target

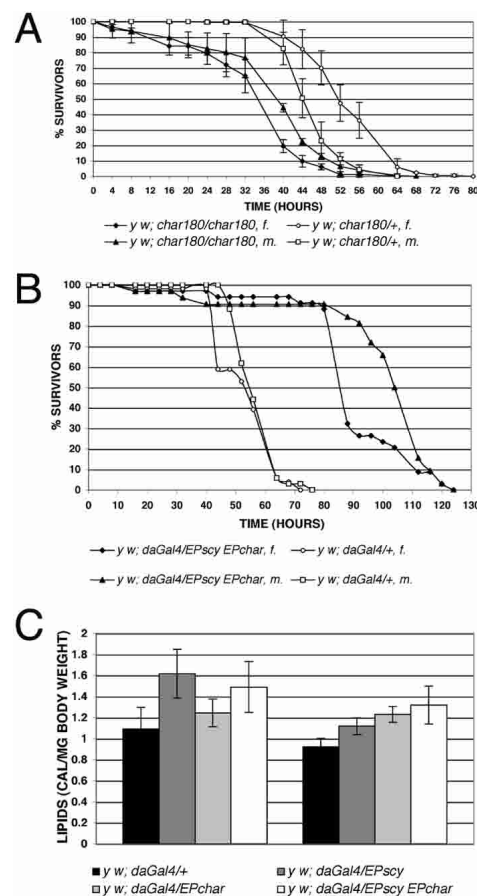


Figure 6. Scylla and Charybdis prolong life span under starvation conditions. (A) Homozygous *char¹⁸⁰* mutants are more susceptible to starvation than control animals. The average and standard deviations of three independent experiments are shown ($n = 114$ for *char¹⁸⁰/char¹⁸⁰, m.*; $n = 145$ for *char¹⁸⁰/+, m.*; $n = 82$ for *char¹⁸⁰/char¹⁸⁰, f.*; and $n = 127$ for *char¹⁸⁰/+, f.*). (B) Two- to three-day-old adult flies coexpressing *scylla* and *charybdis* were starved, and survivors were counted every 4/8 h. Simultaneous overexpression of both proteins strongly extended life span compared to control flies. A representative example of three independent experiments is shown. (C) Ubiquitous expression of *scylla*, *charybdis*, and/or both together using the *daGal4* driver is accompanied by elevated lipid levels in males (left bar cluster) and females (right bar cluster; $p < 0.065$ for *daGal4/EP1035, m.*; for all other genotypes $p < 0.0009$; $n = 10$). (m.) Males; (f.) females.

of *Drosophila* HIF-1 (the Tango-Similar dimer) and that *scylla* and *charybdis* are up-regulated under hypoxic conditions like their mammalian homolog RTP801/REDD1. Furthermore, we show that Scylla negatively regulates Inr/TOR signaling by reducing S6K but not PKB activity.

scylla/charybdis and their orthologs are stress-induced genes

RTP801 was described as a hypoxia/HIF-1-inducible factor with a role in apoptosis (Shoshani et al. 2002). We

find that *scylla/charybdis*, like their mammalian homolog, are induced by hypoxia, and that *scylla* is a direct target of *Drosophila* HIF-1. However, we have no indication that *scylla/charybdis* overexpression promotes cell death. Coexpression of the caspase inhibitors p35 or DIAP1 did not rescue the small eye phenotype induced by expression of either *scylla* or *charybdis* in the eye. Moreover, acridine orange staining revealed no increased cell death upon *scylla/charybdis* coexpression (data not shown). Accordingly, we never observed signs of necrotic eye tissue upon *scylla/charybdis* overexpression.

Dig2, a murine Scylla/Charybdis homolog, is also a stress-responsive protein induced by a variety of treatments including dexamethasone, thapsigargin, tunicamycin, and heat shock (Wang et al. 2003). Together with the finding by Zinke et al. (2002) that *scylla/charybdis* expression is increased during starvation conditions and our analysis, showing that Scylla and Charybdis act as growth inhibitors, these data support a model wherein Scylla and Charybdis, induced by stresses like hypoxia and starvation, act to dampen growth under certain stress conditions.

Do Scylla and Charybdis act together?

Overexpressing either *scylla* or *charybdis* on their own is sufficient to reduce growth. Coexpression of both proteins seemed to have a slight cooperative effect (Fig. 1G) on the PKB/PDK1-dependent eye phenotype with respect to ommatidial structure. Thus, an obvious question is whether Scylla and Charybdis bind to each other and exert their effect only in the presence of the other. Notably, eye-specific *charybdis* overexpression in a *scylla*^{-/-} background resulted in the same phenotype as in a wild-type situation (data not shown). This indicates that Charybdis can act independently of Scylla. This is further supported by their mostly nonoverlapping mRNA expression patterns. Indeed, *charybdis* but not *scylla* is expressed in neuromuscular junctions (M. Knirr and C. Schuster, pers. comm.). Moreover, under hypoxia only *scylla* was induced in the fat body but not *charybdis*, indicating that they may be differentially regulated.

Scylla/Charybdis act downstream of PKB but upstream of TSC to regulate S6K activity

Several lines of evidence indicate that Scylla and Charybdis feed into the Inr pathway downstream of PKB: (1) Scylla antagonizes PKB/PDK1-induced overgrowth in the eye, but in vitro kinase assays demonstrate that Scylla and Charybdis do not reduce PKB kinase activity, nor does the loss of Scylla enhance PKB kinase activity. (2) *scylla* or *charybdis* coexpression can rescue PKB-induced developmental lethality, and ubiquitous *scylla* expression suppresses the lethality associated with hypomorphic *PTEN* mutants. (3) The weight reduction of hypomorphic *PKB*³ flies is partially rescued by the simultaneous absence of Scylla function. (4) Eye-specific PKB/PDK1 expression in a *scylla*^{-/-} background leads to a mild enhancement of the eye phenotype. (5) Overexpression of *PTEN* or *Dp110*^{DN} does not suppress the big

eye phenotype of the tester system, suggesting that our screening system identifies components that act downstream of PKB.

The S6K assay demonstrated that Scylla is capable of reducing S6K activity. Thus, Scylla acts upstream of S6K. This result is consistent with our in vivo results. (1) *scylla* overexpression in the wing reduces wing size by decreasing cell size but not cell number (in fact, cell number is slightly increased), and (2) a *S6K scylla* mutant combination has the same weight as *S6K* single mutants. *S6K* mutants are smaller because of a reduction in cell size but not cell number, making it distinct from other Inr signaling pathway mutants (Montagne et al. 1999). Scylla and Charybdis do not control S6K activity directly but require its upstream regulator TSC. *Tsc1/2* mutants cannot be rescued by overexpression of *scylla*, and the big head phenotype caused by loss of TSC function is not enhanced by the absence of Scylla and Charybdis. Coexpression of *scylla* or *charybdis* and *Tsc1/2* does not further decrease eye size compared to *Tsc1* and *Tsc2* co-overexpression on their own. Moreover, a Rheb-dependent big eye phenotype or lethality induced by ubiquitous *Rheb* expression cannot be suppressed by *scylla* expression. These results indicate that Scylla regulates S6K activity by acting upstream of Tsc1/2 and Rheb. This function appears to be conserved between mammals and flies because RTP801/REDD1 can reduce S6 phosphorylation only in the presence of TSC (Brugarolas et al. 2004).

Our findings indicate that *scylla/charybdis* overexpression mainly affects the TSC/TOR/S6K branch of the pathway downstream of PKB. The PKB-FOXO axis appears not to be influenced by Scylla and Charybdis. Eye-specific overexpression of *scylla/charybdis* in conjunction with *FOXO* was unable to induce *4EBP* (Supplementary Fig. S2). In contrast, simultaneous expression of *FOXO* and *PTEN* or a dominant-negative form of PI3K led to a strong induction of the reporter gene (Junger et al. 2003).

Consistent with an interplay between the Inr and TOR/S6K pathways, *Inr* lethality is suppressed by heterozygosity of *Tsc1* (Gao and Pan 2001). Furthermore, overexpressed PKB phosphorylates and inactivates Tsc2 and thereby activates S6K (Inoki et al. 2002; Manning et al. 2002). Our finding that *scylla* overexpression is sufficient to rescue the lethality associated with *PKB* overexpression indicates that the lethality caused by *PKB* overexpression is due to the hyperactivation of the TOR/S6K pathway. Thus, oncogenic activation of PI3K/PKB signaling seems to be mainly mediated by TOR/S6K signaling. This may explain the beneficial effect of Rapamycin treatment (or its derivatives CCI-779 and RAD001) on *PTEN*-deficient tumors or cells overexpressing *PKB* (Neshat et al. 2001; Podsypanina et al. 2001; Shi et al. 2002; Majumder et al. 2004).

The TSC/TOR pathway as an integrator of growth and metabolism

TSC and TOR receive multiple inputs reflecting the metabolic state of the cell (Hay and Sonenberg 2004).

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AMP-activated kinase (AMPK) is a heterotrimeric kinase that is activated by high AMP/ATP ratios in the cell (Carling 2004). ATP depletion induces Tsc2-phosphorylation, and it was found that AMPK could interact with and phosphorylate Tsc2 (Inoki et al. 2003b). Interestingly, loss of Tsc2 in MEFs and U2OS osteosarcoma cells under low serum and prolonged hypoxia conditions results in HIF-1 α accumulation and concomitantly increased expression of HIF-1 targets in a Rapamycin-dependent manner (Brugarolas et al. 2003). Arsham et al. (2003) showed that mTOR is regulated by decreased oxygen concentration resulting in a dephosphorylation of mTOR at Ser 2481, an mTOR autophosphorylation site. This effect was accompanied by reduced S6K phosphorylation but did not correlate with changes in adenine nucleotide levels and AMPK phosphorylation. Hence, these findings suggest a role for AMPK/Tsc2/mTOR in the integration of oxygen sensing/energy metabolism and growth.

Model of Scylla/Charybdis function

Frei and Edgar (2004) found mutations in the gene encoding *Drosophila* HIF-1 prolyl hydroxylase (Hph), the enzyme rendering HIF-1 α a substrate for proteasomal destruction under normoxic conditions, as dominant suppressors of a Cyclin D/Cdk4-induced bulging eye phenotype. Cells defective for *hph* show a growth deficit, and its overexpression stimulated growth. The authors suggested that the growth-promoting function of Hph is independent of HIF-1 α /Sima. Our results raise the possibility that the Sima target *scylla* is important under hypoxia for growth inhibition.

We have shown that directed expression of Tgo-Sima in the fat body induces *scylla* expression. That this regulation is physiologically relevant can be inferred from three findings. First, *scylla* is also induced under hypoxic conditions. Second, directed expression of other bHLH-PAS proteins like Tgo-Trh or Sim alone did not induce *scylla* expression. Third, survival of flies lacking *scylla* and *charybdis* function is severely compromised under hypoxic conditions. Figure 7 summarizes our current model of Scylla and Charybdis function. *scylla* and *charybdis* are induced in response to external stress stimuli (e.g., hypoxia and starvation) to inhibit growth downstream of PKB but upstream of Tsc1/2. Scylla suppresses growth by reducing S6K activity. This could be achieved by relieving the inhibitory effect of PKB on Tsc2. Alternatively, Scylla/Charybdis could be negatively regulated targets of PKB. This is unlikely, however, since Scylla and Charybdis lack PKB consensus phosphorylation sites. AMPK, activated by drops in energy levels, may also contribute to the induction process of *scylla* and *charybdis* for growth inhibition, presumably under prolonged stress exposure. However, it is also possible that AMPK is controlled by Scylla and/or Charybdis. AMPK decreases protein synthesis by inhibition of S6K in a Rapamycin-sensitive manner, suggesting that mTOR is involved in mediating AMPK signaling (Kimura et al. 2003). AMPK also phosphorylates Tsc2, an

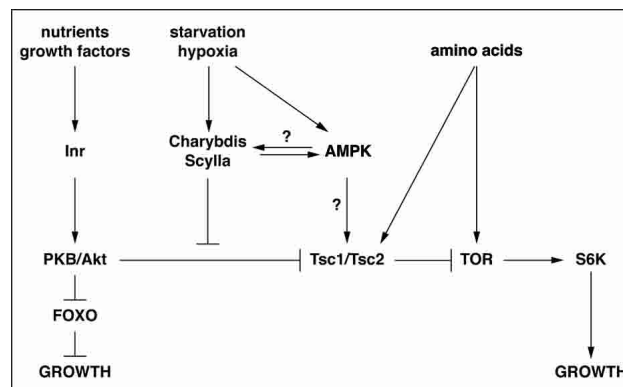


Figure 7. Model of *scylla/charybdis* regulation and their effects. *scylla* and *charybdis* are stress-induced, negative growth modulators that inhibit growth under adverse environmental conditions. They are likely to feed into Inr/TOR/S6K signaling downstream of PKB but upstream of TSC to relieve the inhibitory effect of PKB on the TSC complex, thereby inhibiting growth. Our results do not exclude the possibility that S6K activity also responds to stress independent of Scylla/Charybdis function. Furthermore, Scylla/Charybdis may also regulate or be regulated by AMPK. See text for details.

event important for the cellular energy response pathway (Inoki et al. 2003b).

RTP801/REDD1 may act as a hypoxia-dependent tumor suppressor

In tumors, hypoxic microenvironments are often encountered. Tumor hypoxia is associated with poor prognosis and resistance to radiation-induced cell death. Mutations in the tumor suppressor von Hippel-Lindau (VHL), the subunit of an E3 ubiquitin ligase complex that recognizes proline-hydroxylated residues in HIF-1 α , lead to the formation of a variety of tumors including clear cell carcinomas of the kidney, pheochromocytomas, and hemangioblastomas (Safran and Kaelin 2003). VHL-defective tumors exhibit increased HIF-1 α expression. The induction of *RTP801/REDD1* in cells exposed to hypoxia in tumors raises the possibility that these genes may play a role in tumor development. *RTP801/REDD1* may act as a tumor suppressor. Cells having lost *RTP801/REDD1* function may not stop growing under hypoxic conditions and hence risk accumulating further mutations that promote their tumorigenic state. The analysis of *RTP801/REDD1* expression or mutations in a variety of tumor cell lines should help to test this hypothesis.

Materials and methods

EP element mapping and generation of transgenic flies

The insertion sites of *EP9.85* and *EPscy* were determined by plasmid rescue. To obtain *UAS-scy*, we PCR-amplified a fragment containing the coding region of *scylla* out of a reverse-transcribed embryonic RNA pool (kindly provided by K. Nairz, University of Zurich, Switzerland); all primer sequences available on request). The PCR fragment was subcloned into the pCRII-TOPO vector using the TOPO TA cloning kit (Invitro-

gen). The *scylla* cDNA was cut out of pCRII-Topo as an Asp718/XbaI fragment and inserted into the pUAST vector (Brand and Perrimon 1993) to yield pJR3. At position 414 of the coding region, our sequence differs from that submitted by S. Chauvet, C. Maurel-Zaffran, R. Miassod, N. Julliens, J. Pradel, and D. Aragnol (submitted sequence to the EMBL GenBank DDBJ databases) and results in an A → G exchange. However, the codon still codes for Glu and represents either a silent mutation or a polymorphism.

The shortened *scylla* construct *UAS-scy*^{Δ1–12} was PCR-generated by using pJR3 as the DNA source with an ATG translational start site introduced by PCR mutagenesis. *EP9.85*, inserted 36 bp after the predicted translational start, shows the same effects as *EPscyl*, which lies further upstream of the *scylla* transcription start site. We consider it likely that the last 3 nt of *EP9.85* (i.e., ATG) serve as starter methionine to which amino acid 13 of the original Scylla protein is connected in frame.

To generate the *UAS-char* transgene, we ordered EST LP03309 (Research Genetics) corresponding to *charybdis* and checked the sequence for mutations. *charybdis* cDNA was excised from pOT2 as an EcoRI/XhoI fragment and inserted into the pUAST vector.

Transgenic flies were generated by P element-mediated germline transformation. Constructs were injected into *y w* embryos. At least two independent lines could be established for each construct.

Generation of mutants

For the generation of *scylla* mutants, we mobilized *EP9.85* (marked with *y*⁺) inserted in the *scylla* ORF by supplying Δ2–3 transposase. Jump starter males were mated with *y w*; *D/TM3* females, and single F1 *y*[−] males were crossed to *y w*; *D/TM3* virgins. Approximately 400 stocks were established, and each stock was molecularly investigated for deletions by single-fly PCR using a panel of different primer pairs. Besides the mentioned *scy*³¹ and *scy*¹¹³ deletions, we found three additional smaller deficiencies.

*char*¹⁸⁰ was identified in a local hop screen, mobilizing the original *w*⁺-marked *EPchar* insertion (causing a faint orange eye color). After the mobilization, we scored animals having a darker *w*⁺ eye color than *EPchar* and allowed recombination to occur by crossing single females to *y w* males. Only lines in which all the offspring consistently displayed the darker eye color (i.e., where presumably no recombination has occurred) were further analyzed by PCR using primers that would amplify the *charybdis* genomic locus (but not when a new EP had inserted). Out of 331 single females showing a darker eye color, 112 lines were established and analyzed by single-fly PCR. Only *char*¹⁸⁰ was identified (contains the original *EPchar* element plus a new inverted EP insertion [indicated as *EP1035** in Fig. 1F] ~450 bp upstream of the *char* ORF). Quantitative RT-PCR of homozygous *char*¹⁸⁰ flies revealed a 75% reduction in mRNA levels [Q RT-PCR data available on request].

Fly stocks

The following fly stocks and transgenes have been used in the course of this study: *GMR-Gal4* (gift of M. Freeman, MRC Laboratory of Molecular Biology, Cambridge, UK); *UAS-PKB*, *UAS-PDK1*, *EP837*, and *GMR > w⁺ > Gal4* (Rintelen et al. 2001); *UAS-PTEN* (Huang et al. 1999); *UAS-Dp110*^{DN} (Leevers et al. 1996); *Act5CGal4*, *Lsp2Gal4*, *ZCL0611* (*Scylla* fly trap) and *Df(3L)vin4* (all from the Bloomington *Drosophila* Stock Center); *EP1035* (from the Szeged *Drosophila* Stock Center); *eyflp*; *FRT80 w⁺ c3L/TM6B* (Newsome et al. 2000); *akt*³ (Stocker et al.

2002); *S6K*^{L1}/*TM6B* and *S6K*⁰⁷⁰⁸⁴/*TM6B* (Montagne et al. 1999); *PTEN*^{2L100}/*CyO*, *y*⁺, and *PTEN*^{2L117}/*CyO*, *y*⁺ (Oldham et al. 2000, 2002); *PTEN*^{dj189}/*CyO*, *y*⁺ (Gao et al. 2000); *Tsc1*^{Q87X}/*TM6B*, *y*⁺ (Tapon et al. 2001); *Tsc1*^{2G3} (H. Stocker, unpubl.); *Tsc2*⁵⁶/*TM6B*, *y*⁺, and *Tsc2*¹⁹²/*TM6B*, *y*⁺ (Ito and Rubin 1999); *UAS-tgo*, *UAS-trh*, and *UAS-sim* (gifts of B. Shilo, Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel); *UAS-sima*^{Δ692–863} (Lavista-Llanos et al. 2002); *daGal4/CyO* (A. Wodarz, unpubl.); *Thor*¹ (Bernal and Kimbrell 2000).

All crosses were performed at 25°C if not stated otherwise.

Phenotypic analyses

Freshly eclosed adult flies were collected, separated according to sex, and placed for 24 h on normal fly food before weighing. Weighing was performed using a Mettler Toledo MX5 micro-balance.

scylla-overexpressing wings were mounted in Euparal Mounting solution (TAAB Laboratories). Wing area was determined using Photoshop and NIH Image 1.61 to count pixels. Relative cell number and cell size was determined by extrapolating the counted hairs in a defined area (0.1 mm)² on the dorsal side of the wing proximal to the posterior cross-vein and posterior to vein five.

Adult flies used for scanning electron microscopy were stored in 70% acetone before they were critical-point dried and coated with gold to be examined under the scanning microscope.

Adult fly heads were cut in half using a razor blade and shortly stored in Ringers on ice. Eyes were then fixed as described in Basler et al. (1991).

Heat-shock-induced Gal4 overexpression clones (*y w hsflp*; *GMR > w⁺ > Gal4*, where > corresponds to *FRT* sites) were induced 24–48 h after egg laying (AEL) by a 1-h heat shock at 37°C. The heat shock induces *FLP/FRT*-mediated mitotic recombination leading to the excision of the *w*⁺ marker. *scylla charybdis* double-mutant clones were induced 24–48 h AEL by a 30-min heat shock at 38°C.

Starvation experiments and lipid analysis

Freshly eclosed flies were separated according to sex, left on normal food for 2 d, and subsequently transferred to empty plastic vials sealed with a water-soaked foam stopper. Dead flies were counted every 4/8 h. Determination of lipid and glycogen levels was done essentially as previously described (Bohni et al. 1999).

Hypoxia treatment

Larvae were exposed to the indicated O₂ concentration at room temperature or at 25°C in a closed chamber connected to a gas-mixing pump (Digamix gas mixing pump, type M302 a-F, H Wösthoff e.H.G) that mixes air and N₂, or to a ventilation pump (VENT2 pump, EMKA Technologies) that pushes a nitrogen/air mixture into the chamber. Dissection and fixation of larval tissues for mRNA in situ hybridizations and immunostainings were carried out immediately after hypoxia exposure. *Scylla* protein trap-larvae were dissected in Ringers, and larval tissue was fixed in 4% paraformaldehyde/PBS for 1 h by gentle agitation at room temperature. After a subsequent washing step, nuclei were stained by incubation for 10 min in DAPI (0.5 μg/mL)/PBS followed by three washing steps with PBS. Fluorescence images were recorded using a Zeiss Axiophot microscope.

mRNA in situ hybridizations were done essentially as described (Lehmann and Tautz 1994; O'Neill and Bier 1994). In vitro transcription was done using the DIG RNA labeling kit (Roche). DNA fragments corresponding to parts of the *scylla*

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and *charybdis* full-length mRNAs were PCR-amplified and subcloned into pCRII-TOPO vector. For the in vitro transcription, SP6 and T7 polymerases were used to produce sense and anti-sense RNAs.

PKB-/S6K in vitro activity assays and Western blotting

PKB assay: For each sample, 50 heads of 2–3-d-old female flies were removed with a forceps and collected on a cooled aluminum block placed on dry ice before the heads were transferred to Eppendorf tubes and flash-frozen in liquid nitrogen. Heads were squashed in 400 μ L of extraction buffer (Stocker et al. 2003) using a plastic pestle and subsequently treated as described previously (Radimerski et al. 2002b). One-hundred micrograms of total protein was used for the PKB assay.

For the S6K assay, second instar larvae were collected by floatation in 30% glycerol. Following a brief wash in water, batches of 20–30 larvae were transferred to 1.5-mL Eppendorf tubes and flash-frozen in liquid nitrogen. The S6K assay was done essentially as described (Oldham et al. 2000). Either 25 or 40 μ g of total protein was used for the S6K assay with H2B (Histone type VII-S, Sigma: H4255) as a substrate. Protein concentrations were determined using the RC DC Protein Assay (Bio-Rad).

³²P incorporation into Crosstide or H2B was quantified and background-corrected using a PhosphorImager and image-quant software (Molecular Dynamics).

Western blotting: The following antibodies and dilutions were used: S6K D-20 antibody (Montagne et al. 1999) at 1:200 dilution, PKB antibody (Andjelkovic et al. 1995) at 1:1000 and mouse anti- α -tubulin (Sigma) at 1:2000. HRP-conjugated secondary antibodies (Dako A/S, Glostrup) were diluted 1:2000. Since the same PKB antibody used in immunoprecipitation was also used in Western blotting, HRP-conjugated protein-A (ICN Biomedicals) at 1:5000 was used instead of secondary antibody. Signals were detected using ECL Western blotting detection reagents (Amersham Biosciences).

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