Article

Functional Genomics Identifies TOR-Regulated Genes that Control Growth and Division

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Summary

Background: The TOR (target of rapamycin) ser/thr protein kinase is the central component of a eukaryotic signaling pathway that regulates growth and is the direct target of the clinically useful drug rapamycin. Recent efforts have identified at least two multiprotein complexes that contain TOR, but little is known in higher eukaryotes about the genes downstream of TOR that control growth.
Results: By combining the use of a small molecule inhibitor (rapamycin), transcriptional profiling, and RNA interference in Drosophila tissue culture cells, we identified genes whose expression responds to Drosophila TOR (dTOR) inhibition and that regulate cell size. Several of the dTOR-regulated genes that function in cell size control have additional roles in cell division. Most of these genes are conserved in mammals and several are linked to human disease. This set of genes is highly enriched for regulators of ribosome biogenesis, which emphasizes the importance of TOR-dependent transcription in building the protein synthesis machinery in higher eukaryotes. In addition, we identify two dTOR-regulated genes, CG3071 and CG6677, whose human orthologs, *SAW* and *ASH2L*, are also under TOR-dependent transcriptional control and encode proteins with conserved functional roles in growth.
Conclusions: We conclude that combining RNA interference with genomic analysis approaches, such as transcriptional profiling, is an effective way to identify genes functioning in a particular biological process. Moreover, this strategy, if applied in model systems with simpler genomes, can identify genes with conserved functions in mammals.

Introduction

Cell growth is the process by which cells accumulate mass. mTOR, a large ser/thr protein kinase and the direct target of the clinically important drug rapamycin, is a critical and conserved regulator of growth [1]. Studies in yeast, Drosophila, and mammalian cultured cells

indicate that mTOR functions in two distinct complexes called mTOR complex 1 and 2 (mTORC1 and mTORC2), respectively [2–8]. Both complexes contain mTOR and a small WD-repeat protein GbetaL (GβL), but their substrate specificities are determined by interactions between mTOR and two unique accessory proteins, Raptor and Rictor. mTORC1, which is sensitive to rapamycin, contains Raptor and controls cell growth. mTORC2, which is bound by Rictor, is not a direct target of rapamycin and is predicted to have a role in cell survival and cell proliferation.

mTORC1 lies downstream of the *TSC1* and *TSC2* genes, which are mutated in TSC (Tuberous Sclerosis Complex) and the related disease LAM (Lymphangioleiomyomatosis) [9]. The TSC1 and TSC2 proteins form a two-component GTPase activating protein (GAP) complex that inhibits mTORC1 growth signaling through a small GTPase called Rheb in response to nutrient status, energy availability, stress, and growth factors [1]. One well-described downstream target of mTORC1 is the S6 kinase 1 (S6K1) [1]. mTORC1 activates S6K1 by directly phosphorylating it in a C-terminal hydrophobic motif. mTOR knockout models in Drosophila and mice are early lethal, but in contrast, knockout models of S6K1 are viable and reduced in size, suggesting that S6K1 contributes to the growth response but other outputs of mTORC1 are important [10–16]. mTORC1 also phosphorylates and inhibits the eIF-4E binding protein 1 (4E-BP1). eIF-4E, which is inhibited through direct binding of 4E-BP1, promotes cap-dependent translation. In Drosophila and mice, 4E-BP1 is dispensable for viability and in Drosophila 4E-BP is required under stressful conditions to halt metabolism and promote survival [17, 18]. An important inhibitory role for mTORC1 in autophagy, a cellular process of self-digestion, is also emerging [19].

The rate of cell growth correlates with ribosome number in addition to the efficiency of translation initiation. In yeast, it is the combined rate of rRNA and ribosomal protein transcription that determines the rate of ribosome biogenesis [20]. It is also well established in yeast that TOR-dependent control of protein synthesis involves transcriptional regulation of ribosome biogenesis genes [21, 22]. Yeast TOR modulates ribosome biogenesis through controlling rRNA and ribosomal protein transcription [21–23]. The transcription of a large number of genes called the *Ribi* regulon, many of which encode ribosome assembly factors [24–28], is also influenced by TOR signaling in yeast [29]. mTOR-dependent transcriptional regulation of rRNA in mammalian cells is rapamcyin sensitive [30–33], but whether mTOR signaling coordinates rRNA transcription with transcription of ribosomal proteins and ribosome assembly factors is still unclear.

Recent work revealed that the mTORC1 growth pathway is hyperactive in many cancers, propelling rapamycin into clinical trials as a treatment for certain cancers, TSC, and LAM [1, 9]. One model suggests that loss of
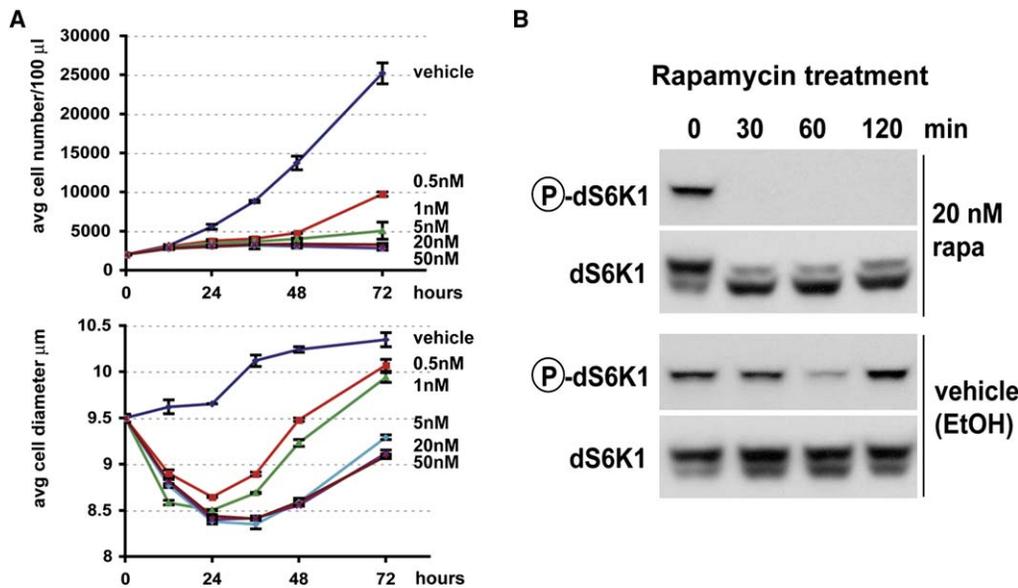
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Figure 1. Growth Is Inhibited by Rapamycin in *Drosophila* S2 Cells

(A) *Drosophila* S2 cells were treated with rapamycin at the indicated concentrations. A time course experiment indicates that S2 cells decrease in size and arrest division within 24 hr of rapamycin treatment. By 72 hr post rapamycin addition, the cell size defect partially recovers but cells remain arrested. The average value and standard deviation for three independent measurements are shown for each data point.

(B) Rapamycin treatment results in rapid and complete dephosphorylation of dS6K1 T398, which is equivalent to the mTOR-dependent T389 hydrophobic motif site in mammalian S6K1. Ethanol, the rapamycin solvent and vehicle control, reproducibly decreases dS6K1 phosphorylation modestly at 60 min, consistent with reports that alcohol can impair phosphorylation of S6K1 [41, 42].

PTEN function promotes Akt/PKB-dependent inhibition of TSC2 and activation of mTORC1 [34–36], although the physiological significance of this event has been questioned [37]. The prevailing view is that an activated mTORC1 promotes protein synthesis through S6K1 and 4E-BP1 and gives tumors a growth advantage. Transcriptional profiling in yeast and mammalian cells indicates that mTORC1 additionally regulates a diverse array of genes that could also impact tumor progression [21, 38–40]. mTORC1-responsive genes could be utilized as biomarkers of mTOR activity as well as potential targets for antigrowth drugs.

Considering the significance of mTOR signaling in cancer, we devised a strategy that combines gene-expression profiling with RNAi-based loss-of-function analysis to screen for genes regulated by TOR that function in growth control. To improve the probability of finding growth genes, we first identified a set of genes whose transcription is rapidly responsive to *Drosophila* TORC1 (dTORC1) inhibition by acute rapamycin treatment of cultured cells. We next determined the functional role of each dTOR-regulated gene in cell size control by RNAi. This approach identified several regulators of cell growth, some of which also have roles in cell division control. A significant proportion of the identified genes function in multiple steps along the ribosome assembly pathway, emphasizing the intimate link between ribosome biogenesis and cell size regulation and suggesting that in higher eukaryotes as in yeast, TOR has a critical and complex role in controlling protein synthesis on many different levels. Finally, we identified dTOR-regulated growth genes that are also transcriptionally regulated in mammalian cells by mTOR and that have a conserved functional role in growth.

Results

Identification of Genes Responsive to Acute Rapamycin Treatment

To search for genes regulated by the TOR kinase, we used *Drosophila* cultured S2 cells as a model system because the TOR pathway is highly conserved in *Drosophila*, the *Drosophila* genome is less redundant than mammals, and importantly for functional studies, these cells are particularly amenable to RNAi. TOR bound to Raptor is inhibited by rapamycin and controls growth, which can be determined empirically by measuring cell size. A time course experiment indicates that S2 cells decrease in size and arrest division within 24 hr of rapamycin treatment at concentrations between 1 and 50 nM (Figure 1A). By 72 hr post rapamycin addition, the cell size defect partially recovers but cells remain arrested. As expected, rapamycin treatment results in rapid and complete dephosphorylation of dS6K1 T398, which is equivalent to the mTOR-dependent T389 hydrophobic motif site in mammal S6K1 (Figure 1B). Ethanol, the rapamycin solvent and vehicle control, reproducibly decreases dS6K1 phosphorylation modestly at 60 min, consistent with reports that alcohol impairs phosphorylation of S6K1 [41, 42].

To identify genes regulated by dTOR, we generated gene expression profiles of cultured S2 cells after 0, 30, 60, or 120 min of rapamycin treatment. 428 and 588 genes exhibited patterns of decreased or increased expression, respectively, after the time course (see Figure S1 in the Supplemental Data available with this article online). Of the genes significantly changing expression (p < 0.01), 35% and 42%, respectively, had gene ontology (GO) annotations. Overrepresented among

the upregulated genes are genes functioning in cell-cycle control, Pol II transcription, programmed cell death, DNA metabolism, and response to stress (Figure S2). Downregulated genes are overrepresented for genes functioning in protein folding, ribosome biogenesis, electron transport, protein targeting, oxidative phosphorylation, mRNA processing, and protein transport.

We reasoned that the set of genes significantly responsive to rapamycin might have a high probability of containing dTOR-regulated genes that function in growth and/or division control. From the list of genes differentially expressed after rapamycin treatment, we identified a subset of 84 genes that significantly ($p < 0.01$) increase or decrease expression 2-fold or more at each time point (Figure S1). GO analysis of this filtered gene set indicates that of the overrepresented genes decreasing in expression, many function in Pol I transcription, rRNA metabolism, ribosome biogenesis, and protein folding, while transcription regulators are overrepresented among genes increasing in expression (Figure S3).

dTOR-Regulated Genes Function in Growth Control
We hypothesized that many of the significant dTOR-regulated genes identified by expression profiling would function in controlling cell growth. To explore this possibility, we first established that RNAi-mediated silencing of several dTOR pathway components in *Drosophila* S2 cells causes dramatic changes in cell growth as determined by measuring individual cell size (Figure 2A). For instance, RNAi-mediated silencing of dTOR or dRaptor results in a reduced mean cell diameter and a corresponding decrease in dS6K phosphorylation (data not shown), a phenotype reminiscent of rapamycin treatment. Likewise, silencing dRheb decreases cell diameter to a size comparable to dTOR- or dRaptor-silenced cells, supporting the prediction that Rheb activates TORC1. RNAi-mediated silencing of dS6K causes an intermediate reduction in cell size, which is consistent with genetic studies. Similarly, silencing dAKT also reduced cell size. Conversely, silencing the dTSC2 or dPTEN tumor suppressors increases mean cell diameter, consistent with their role as negative growth regulators. We converted individual cell size distribution curves to one-dimensional stacked images for visualizing many cell size curves simultaneously (Figure 2B).

Since the cell size decrease resulting from knockdown of dTOR, dRaptor, or dRheb more closely resembles rapamycin treatment than does knockdown of dS6K (compare Figures 1 and 2), we reasoned that other dTOR outputs might contribute to growth control. One possibility is that the combined efforts of both dS6K and deIF-4E (the negatively regulated target of the TOR substrate 4E-BP1) mediate TOR-dependent growth. Knockdown of either dS6K1 or deIF-4E reduces mean cell diameter to a similar degree (Figure 2C); however, silencing both dS6K1 and deIF4-E together does not reduce mean cell diameter to the same extent as rapamycin treatment (Figure 2C). Although growth is inhibited here by two different mechanisms (drug treatment versus RNAi), this comparison suggests that other dTOR outputs contribute to growth.

We next used RNAi to systematically silence all of the significant rapamycin-responsive genes that changed 2-fold and measured the resultant effects on cell size.

In total, 54 of the genes we screened (~65%) had a cell size defect after RNAi, 49 of which were reproducible with a p value <0.05 (Figure 3 and Table S1). Observed changes in cell size could reflect defects in cell proliferation, so we additionally measured cell number. Genes that reduce cell number to 65% or less of the control cell number when silenced were thus categorized as having both cell-growth and cell-proliferation defects (Figure 3B). Silencing of the remaining genes, in most cases, had a significant effect on cell size while minimally reducing cell number (Figure 2A). A detailed description of the genes identified can be found in Table S2. This analysis identified both positive growth regulators (genes that decrease cell size when silenced) and negative growth regulators (genes that increase cell size when silenced). Importantly, we identified genes that when silenced decrease cell size to the same extent as dTOR RNAi, as well as genes whose silencing led to cell size increases comparable to silencing dTSC2 or dPTEN silencing. There appears to be good correlation between the gene expression response (down or up) and effect on cell size (decrease or increase) such that genes inhibited by rapamycin are generally positive growth regulators, and at least two genes that increase expression seem to be negative growth regulators. Most of these *Drosophila* genes have human orthologs, some of which are linked to diseases (see Table S2 and Discussion).

To confirm that the effects of rapamycin on gene expression reflect an inhibition of the dTOR growth pathway, we compared the gene expression profiles from rapamycin-treated cells with those from cells knocked down for dRheb, dTOR, dRaptor, and dRictor. We did not expect these comparisons to show dramatic overlaps since the analysis compares the effects of an acute drug treatment with those of chronic gene silencing. However, k-means clustering (k = 3) of knockdown expression data revealed that silencing dRheb, dTOR, and dRaptor generated expression profiles resembling rapamcyin treatment (Figure 4A). The dRheb knockdown profile was most similar with a Pearson correlation of 0.40 (p value < 1e−10) when compared to the 120 min drug-treatment profile, followed by dTOR (0.29; p value < 1e−5) and dRaptor (0.17; p value = 0.002). Consistent with dRictor functioning in a rapamycin-insensitive dTOR pathway, there was no correlation between the dRictor and rapamycin profiles (−0.06; p value = 0.9).

To further validate our approach and to test if the genes we identified might be relevant to growth-related diseases, we performed combination knockdown experiments by silencing dTSC2 (a negative regulator of dTOR) in combination with silencing several of the genes found to positively regulate growth. Silencing dTSC2 results in a large increase in mean cell diameter (Figures 2A and 4B); however, the cell size increase was suppressed in each case when we silenced a candidate positive growth gene in combination with dTSC2 (Figure 4B). In most cases, final average cell size in each combination knockdown was close to the control average cell size rather than the average cell size resulting from knockdown of the candidate gene alone. This "equilibration" might represent the fact that gene knockdowns are not equivalent to knockouts and residual protein expression persists. Alternatively, it might reflect that downstream in the

A

Cell size distribution
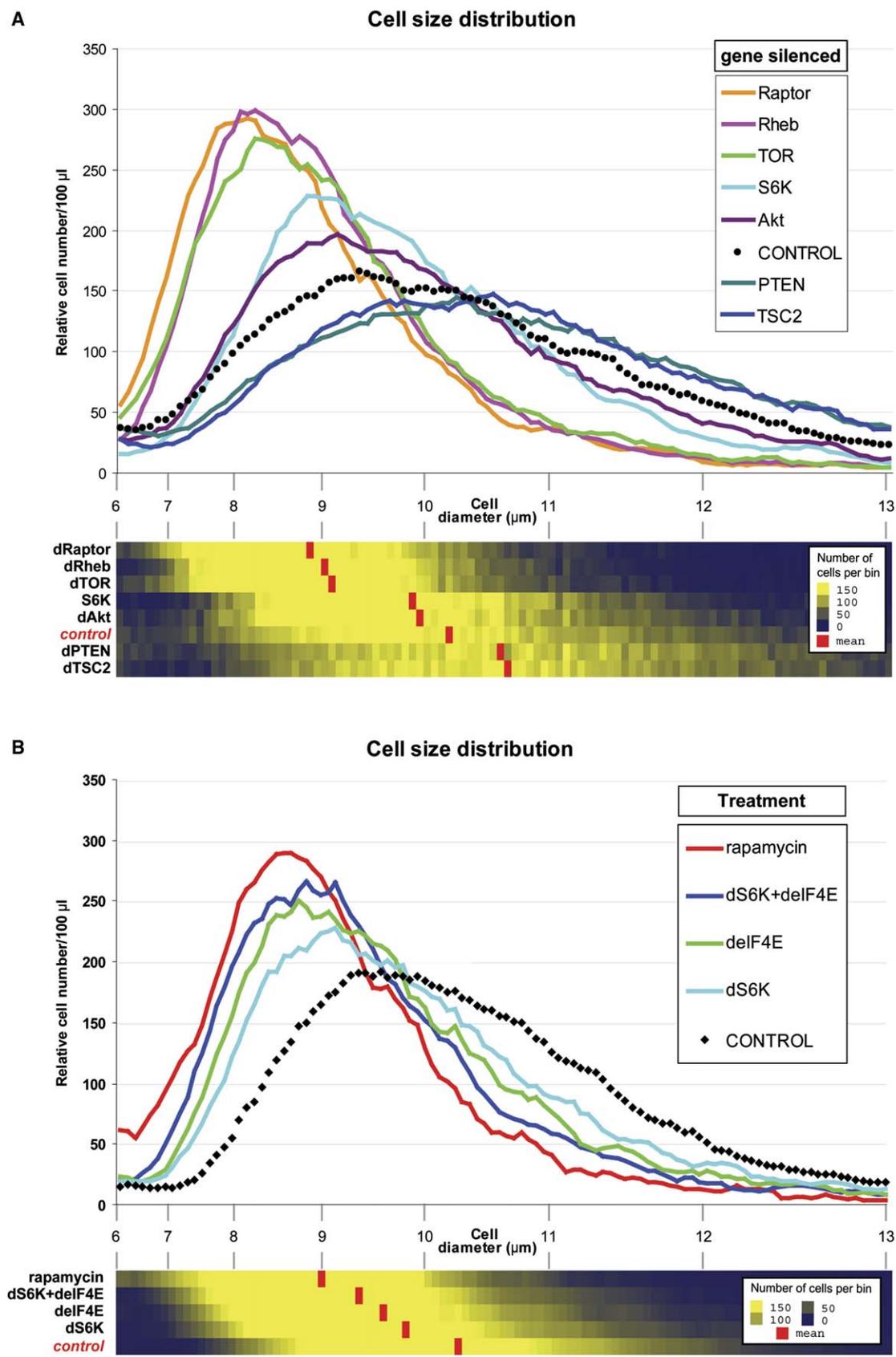

B

Cell size distribution

Figure 2. Cell Size Regulation by the dTOR Pathway

(A) Normalized cell size distributions of S2 cells knockeddown for various positive and negative regulators of the dTOR growth pathway. Target genes are indicated in the box and color coordinated with the appropriate lines on the graph. Cell size data is displayed below as

dTOR growth pathway signaling branches and these candidate genes when silenced might only compromise one output. Regardless, these data are consistent with the identified genes functioning downstream of dTSC2 although it does not rule out that they could function in a parallel pathway.

Rapamycin Decreases Expression of Ribosome Assembly Genes that Control Growth

Since GO analysis of the filtered set of rapamycin-responsive genes revealed that ribosome biogenesis regulators are overrepresented among the known genes, we wondered if any of the uncharacterized genes we identified might also have roles in ribosome biogenesis. We compared the sequence of each unidentified *Drosophila* gene with its closest yeast ortholog by InParanoid V4.0 (http://inparanoid.cgb.ki.se/index.html). 70% of all the genes in Figure 2 have a yeast ortholog with a defined or predicted function (Table S3). Of the genes decreasing expression in response to rapamycin, a striking 69% (33/48) have a budding yeast ortholog predicted or known to function in controlling ribosome assembly. Review of current literature suggests that these genes function at multiple steps of ribosome synthesis including assembly of the 90S preribosomal particle (SSU processome) and assembly of both the 60S and 40S subunit of the ribosome (Figure 4C) [43, 44]. Thus, in *Drosophila* S2 cells, acute rapamycin treatment downregulates expression of genes that function in ribosome assembly pathways and have a role in maintaining proper cell size and cell division control. Although rRNA transcription is sensitive to rapamycin in mammalian cells, a link between ribosome assembly factor transcription and TOR signaling has not been described in higher eukaryotes.

Consistent with the additional role for TOR signaling in regulating rDNA transcription, three genes (CG18600, CG3756, CG4033/*Rpl135*) identified in our functional assay as decreasing expression in response to rapamycin and reducing size when silenced are predicted to encode components of the RNA pol I complex (Figure 4C). The yeast ortholog of CG3756 is also part of the RNA pol III complex. One gene (CG13096) is related to the human Ribosomal L1 domain containing protein 1 and a second gene (CG5033) is predicted to encode ribonucleoprotein involved in ribosome biogenesis, again emphasizing the ribosome as a target for transcriptional control. Four heat shock proteins (CG1242/*hsp83*, CG12101/*hsp60*, CG11267, and CG6603/*hsc70Cb*) were also identified. For unknown reasons, silencing of these genes increased cell size and reduced cell proliferation.

Rapamycin Increases Expression of Transcription Factors that Control Growth

Only four genes increasing in expression 2.0-fold or more (p < 0.01) induced cell size changes when silenced (Figure 3 and Table S1). Since rapamycin inhibits growth, we thought it possible that silencing of these genes might promote cell growth. Only two of the genes (CG4427 and CG6677/*ash2*), both encoding transcriptional regulatory proteins, resulted in an increased mean cell diameter when silenced (Figure 3A). These genes were previously identified together in a screen for regulators of axon guidance and synaptogenesis in *Drosophila* [45], and CG4427 was also identified in a screen for genes involved in autophagic cell death [46]. Interestingly, CG6677/ASH2 silencing increased cell size to the same extent as the silencing of the tumor suppressors dTSC2 or dPTEN, suggesting that it too might have a tumor-suppressor character. Although ASH2 knockdown cells grew to a similar size as dTSC2 and dPTEN knockdown cells, they did so by a unique mechanism because ASH2 silencing had no effect on dS6K or dAKT phosphorylation, two effectors functioning downstream of dTSC2 and dPTEN, respectively (data not shown). In contrast, *stai* and *mu2* appear to reduce mean cell diameter when silenced. The reason for this inverse correlation is unclear but could reflect a dual function for these gene products.

Mammalian Orthologs of CG3071/SAW and CG6677/ASH2 Are Regulated by mTOR and Control Cell Size

We wondered if our approach could identify genes with conserved responses to TOR signaling in mammalian cells. The human orthologs to the genes identified in this study are listed in Table S2. We selected CG3071 based on its conservation as a representative gene that decreased expression in response to rapamcyin and encodes a positive growth regulator. CG3071 is 39% identical (59% similar) to human UTP15/SAW (Src-associated protein with WD repeats; NP_115551.2). We also choose CG6677/*ash2* as a representative gene that increases expression in response to rapamycin and encodes a negative regulator of growth. CG6677/ASH2 is 46% identical (62% similar) to human ASH2L.

Real-time quantitative RT-PCR analysis indicates that the expression of *SAW* decreases over time in HEK-293T cells after 3 hr of rapamycin treatment, reaching a 35% decline in expression by 3 hr (Figure 5A). In contrast, *ASH2L* expression steadily increases after rapamycin treatment, increasing by more than 100% (Figure 5A). We conclude that these effects are due to mTOR inhibition because identical trends in gene expression were obtained when cells were stably knocked down for mTOR via a previously validated lentiviral shRNA expression system [6] (Figure 5B). In these cells, *SAW* levels decreased by nearly 70% and *ASH2L* levels again more than doubled. *mTOR* mRNA levels decrease by more than 90% in cells expressing the mTOR shRNA, indicating robust silencing (Figure 5C). By means of a commercially available antibody to ASH2L, we also observe an increased level of an 80 KDa protein corresponding to ASH2L in HEK-293T and HeLa cell lysates generated from rapamycin-treated cells (Figure 5D). mTOR levels are unchanged during the time course, but growth signaling is inhibited as indicated by phospho-S6K1 levels (Figure 5D). A similar increase in ASH2L protein was observed in mTOR knockdown cells (Figure 5E). We

a one-dimensional stacked distribution of histograms ordered from smallest to largest mean cell diameter. Blue color indicates fewer cells in bin, while yellow color indicates more cells.
(B) Normalized cell size distributions of cells knocked down for dS6K and/or deIF4E compared to rapamycin-treated cells. Note that in both figures the x axis is nonlinear.
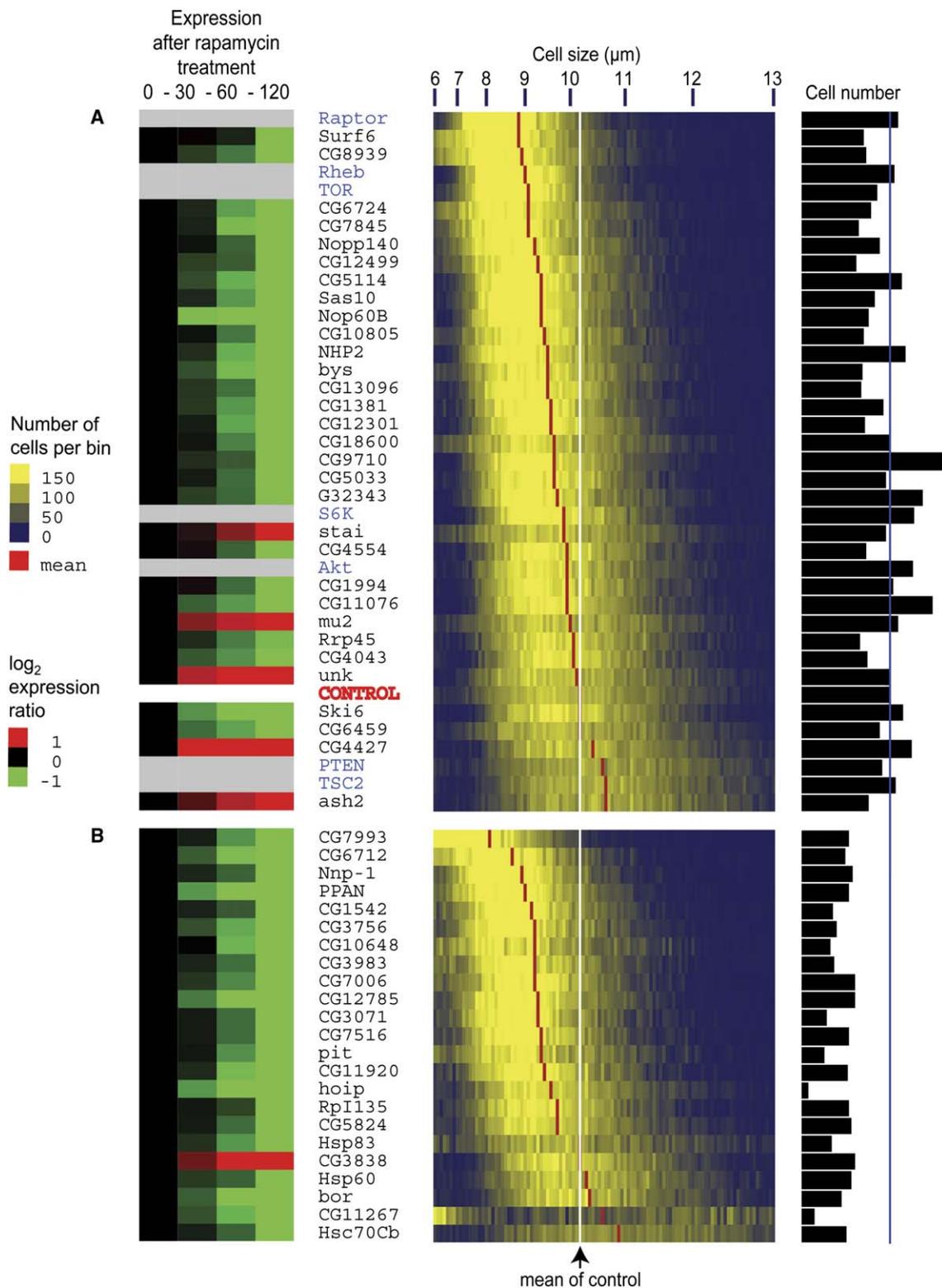
Figure 3. Functional Analysis of dTOR-Regulated Genes

Rapamycin-induced expression profiles and the corresponding cell size distributions after RNAi. The TOR-pathway components described in Figure 1 are included as positive controls and their expression changes have been shaded gray. Cell size distributions are ordered from smallest mean cell diameter (top) to largest (bottom). The corresponding cell number count after RNAi is indicated to the right. We arbitrarily decided that a cell number count less than ~65% of the control qualified as a cell proliferation defect. The mean cell diameter and cell number of the control is marked with a white and blue line, respectively. Knockdowns that induce cell size changes (A) and knockdowns that induce both cell size and cell proliferation changes (B) are separated.
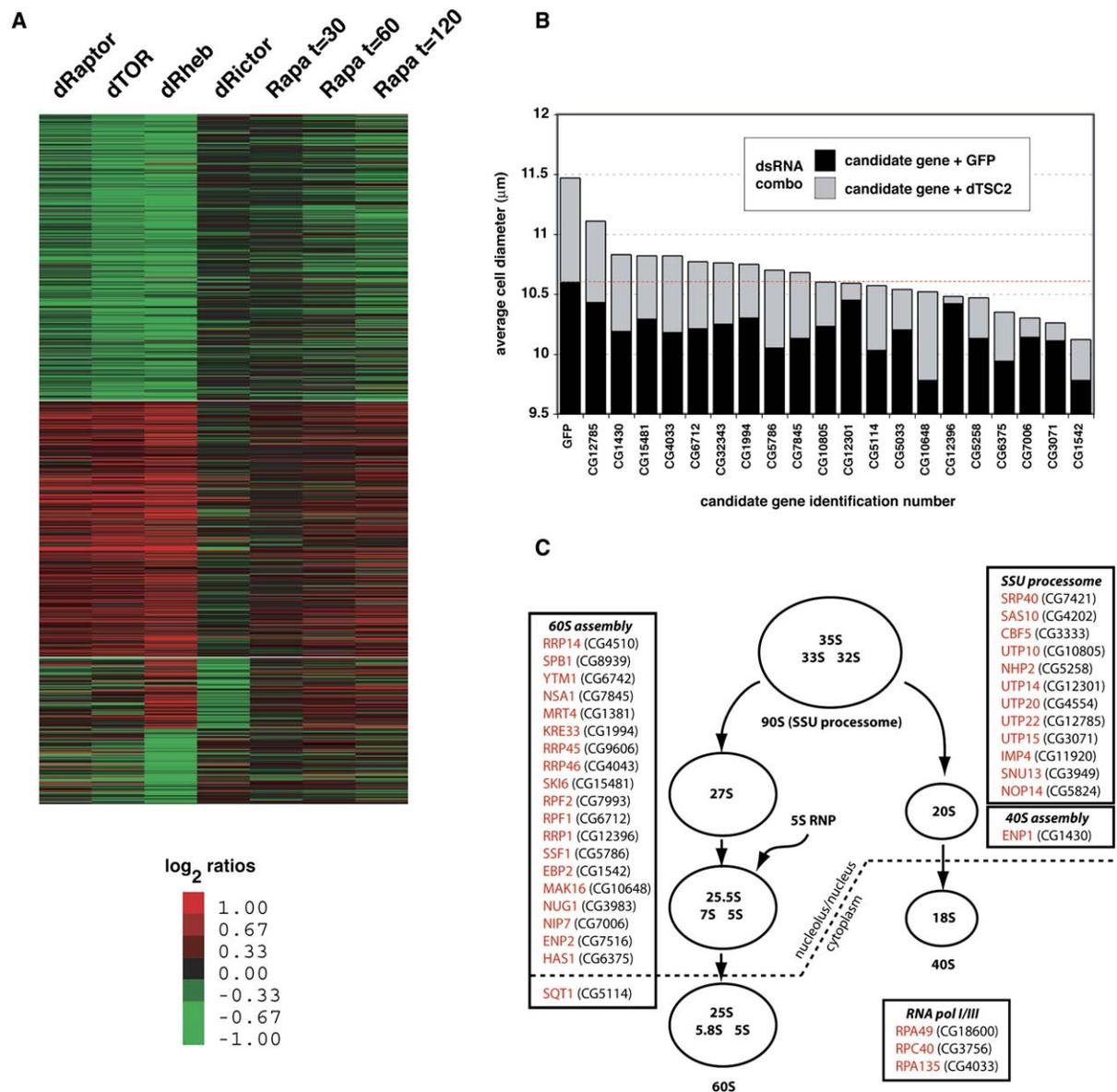
Figure 4. Rapamycin-Regulated Genes Function Downstream in the dTOR Growth Pathway

(A) Gene expression signatures from rapamycin-treated cells are similar to signatures from cells knocked down for dRaptor, dTOR, and dRheb, but not dRictor.

(B) Combination knockdown of candidate genes with dTSC2. We randomly selected 20 genes identified as positive growth regulators by our screen and silenced them by RNAi alone or in combination with the negative growth regulator dTSC2. In all cases, silencing the candidate gene in combination with dTSC2 reduced the cell size increase caused by silencing dTSC2. The dashed red line indicates the average cell size for the GFP control cells.

(C) dTOR-regulated growth genes mediate ribosome assembly. Nearly 70% of the genes identified by our screen have a yeast ortholog that mediates ribosome assembly (yeast genes in red). The yeast/*Drosophila* gene pairs are listed next to the corresponding step in ribosome assembly where they are predicted to function. Also listed are three yeast/*Drosophila* gene pairs identified that function in RNA polymerase I/III transcription.

confirmed that this is ASH2L by reducing its expression greater than 80% with two unique lenti-viral expressed shRNAs (Figures 5C and 5E). This shows that the rapamycin-induced *ASH2L* transcriptional response translates into an increase in ASH2L protein.

We next measured the effect of silencing SAW and ASH2L on cell size. Real-time quantitative RT-PCR analysis indicates that SAW and ASH2L mRNA levels can be reduced by 80%–90% with at least two unique hairpins per gene (Figure 5C). Compared to HEK-293T cells infected with a control shRNA to *GFP*, *SAW* knockdown

cells are reduced to roughly the same size as cells knocked down for *mTOR* (Figure 5F). In contrast, cells expressing ASH2L shRNA hairpins are significantly larger than control cells (Figures 5F and 5G). Interestingly, the cell size increase caused by ASH2L silencing is partially sensitive to rapamycin, supporting the hypothesis that other rapamycin-sensitive outputs work in combination with ASH2L to regulate size (Table S4). Thus, as with their *Drosophila* orthologs, *SAW* and *ASH2L* are transcriptionally regulated by mTOR and have a functional role in growth (Figure 5H).
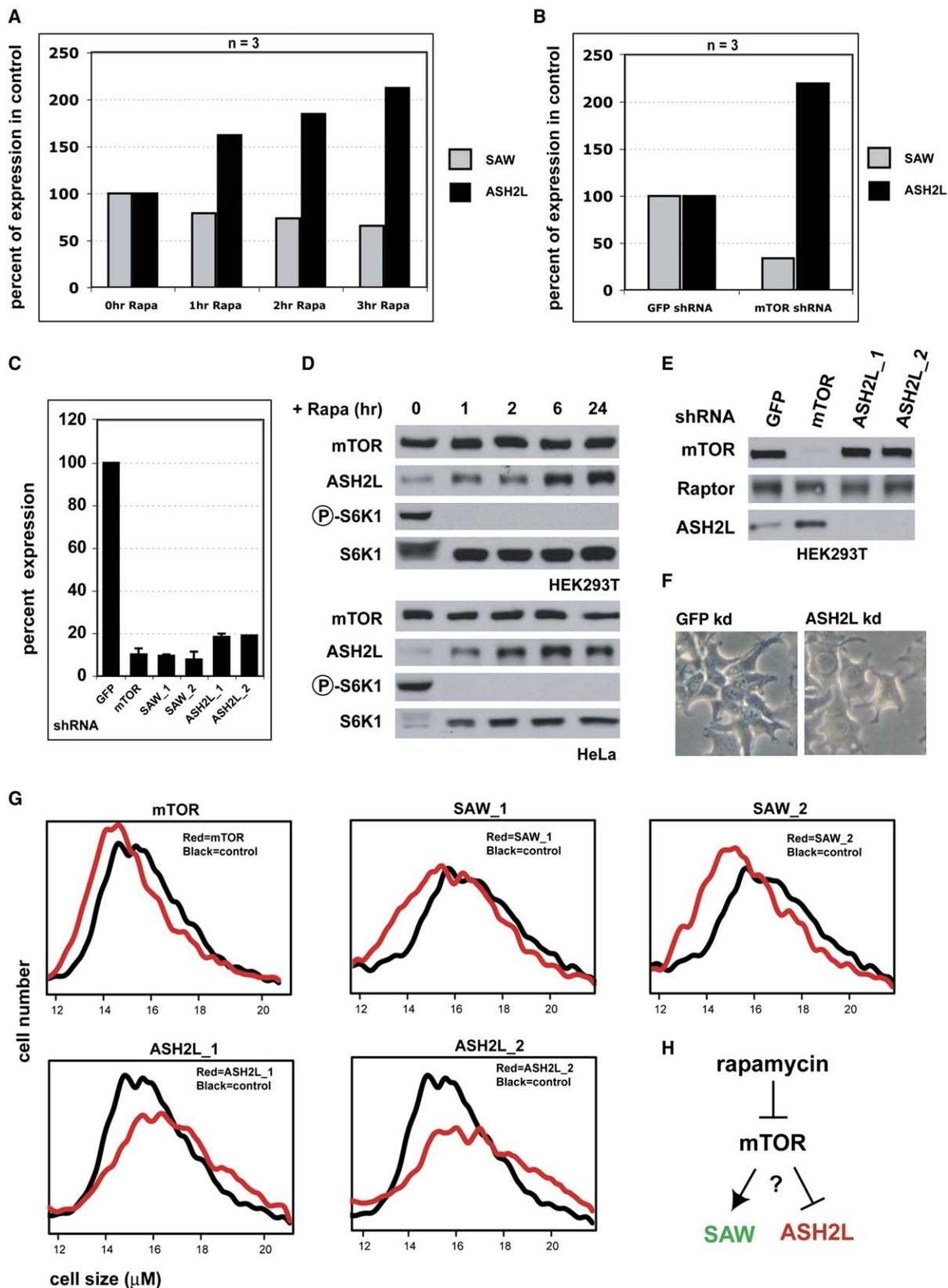
Figure 5. CG3071 and CG6677 Have Mammalian Orthologs, SAW and ASH2L, with Conserved Transcriptional Responses and Function

(A) Real-time quantitative RT-PCR was used to measure changes in *SAW* and *ASH2L* mRNA levels in HEK293T cells after treatment with 20 nM rapamycin for 0, 1, 2, or 3 hr.

(B) Real-time quantitative RT-PCR was performed as in (A) except that total RNA was prepared from HEK293T cells stably expressing lenti-viral shRNAs that target GFP or mTOR for silencing.

Discussion

Identification of TOR-Regulated Growth Genes

We define growth as an increase in cell mass, which is distinct from cell division, although the two processes are coordinated [47]. How coordination is achieved is still debated, but in many cells, cell-cycle progression requires growth. From this model has emerged a hypothesis that a "size threshold" exists that prevents cell-cycle progression until a critical size is attained. When assessing size, it is not clear what cells measure, but the rate of protein synthesis is a good prediction. Therefore, it is not surprising that rapamycin treatment (which is thought to mimic nutrient deprivation) would change the expression of genes with roles in various aspects of setting the protein synthesis rate.

Previous studies in *Saccharomyces cerevisiae* investigating the role of TOR in transcription determined that yeast TOR regulates expression of metabolic pathway genes, and in addition, ribosomal RNA and ribosomal protein genes; transcription, translation, and replication factors; and protein degradation genes [21, 38, 39]. Our previous studies with mammalian cells indicated that rapamycin affects expression of a diverse array of genes functioning in nutrient and protein metabolism [40]. But in these studies, a functional connection to growth was not established. Subsequent work from the Tyers group showed that ribosomal proteins and ribosome assembly factors have a critical role in controlling growth [27]. Furthermore, two proteins (Sfp1 and Sch9—an AGC kinase related to AKT and S6K) that activate expression of transcriptional units encoding ribosomal proteins and ribosome assembly factors are regulated in part by TOR [27, 29]. Our study suggests that in higher eukaryotes, TOR regulates expression of genes functioning in multiple steps in the ribosome assembly pathway. But importantly, we found that many rapamycin-responsive genes that are necessary for normal growth encode ribosome biogenesis regulators, a connection not made in higher organisms.

We further consulted previous genetic studies in *Drosophila* to determine if any of the genes we identified by a cell culture-based system have previously been shown to have a role in organismal growth. One positive growth gene we identified was CG3333/*Nop60B*. Partial loss of *Nop60B* (also named *minifly*) function results in severe reduction in body size and developmental delay [48]. One positive growth and proliferation gene we identified, CG6375/*pitoune/pit*, is also required for cell growth and proliferation in developing *Drosophila* larvae [49]. Finally, CG5786/*ppan* (*peter pan*), a gene identified in a screen for larval growth regulators, was also found to be required for normal growth and proliferation of cultured cells [50]. These findings suggest that our approach led to the identification of physiologically relevant genes.

While our report claims to have separated TOR-regulated growth genes from genes also affecting proliferation, an important point to consider is that "knockdown" by RNAi, while efficient in *Drosophila* cultured cells, does not substitute for a genetic "knockout." RNAi can provide convincing clues to a gene's function, but residual expression of a target could result in an intermediate phenotype. This could be a powerful advantage for functionally annotating genes that would otherwise be essential for viability. However, a cautionary interpretation should be applied when using RNAi as a tool to distinguish pure growth genes (i.e., genes that affect only cell growth) from genes that control growth and proliferation. It is possible that for some genes, knockdown was sufficient to inhibit growth, but perhaps a more robust knockdown would inhibit cell-cycle progression. Furthermore, our study primarily looks at individual mean cell size as the readout for growth, and growth is defined as an increase in mass. For some genes, such as CG11267 and Hsc70Cb, knockdown increased cell size but greatly reduced cell number, thereby reducing total cell mass of the population. Along the same lines, a gene that increases cell number but not cell size would increase the total mass of a cell population. Thus, consideration of the global cell population number in addition to mean cell size after gene silencing can provide more information about a gene's function in growth. However, making these predictions here is again challenged by the incomplete nature of knockdowns versus knockouts. Future studies that use genetic knockout models should address these concerns on a gene-by-gene basis.

Nutrient Control of Gene Expression in Growth and Disease

Nutrient control of gene expression and growth has emerged as a principal concern in the modern era as diet-induced diseases become more prevalent. It is believed that mTOR is at the core of an ancient growth pathway that senses nutrient levels, particularly amino acids, and that rapamycin treatment mimics a "starvation-like" state. A previous study employing Affymetrix microarrays identified a set of genes in developing *Drosophila* larvae that respond to starvation [51]. 42% of the 19 genes identified by Zinke et al. as responding negatively to amino acid starvation were also rapamycin-sensitive genes: a remarkable similarity considering the difference in source material and statistical analysis. If we consider all the genes whose expression decreased significantly ($p < 0.01$) after rapamycin treatment, we find that 68% of the genes in the list of Zinke et al. are also in ours. While none of the genes Zinke et al. identified as

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(C) Real-time quantitative RT-PCR analysis of mRNA in HEK293T cells knocked down for mTOR, SAW, or ASH2L.

(D) ASH2L protein level increases in HEK293T and HeLa cells after rapamycin treatment. Immunoblotting was used to indicate the total amounts of mTOR, ASH2L, S6K1, and phospho-S6K1-T389.

(E) Lenti-viral shRNAs were used to target GFP (control), mTOR, or ASH2L (two unique hairpins shown) in HEK293T cells. Total amounts of mTOR, Raptor, and ASH2L protein after knockdown are shown by immunoblotting.

(F) Representative images of HEK293T cells expressing a GFP (control) or ASH2L shRNA.

(G) Cell-size distributions of HEK293T cells knocked down for mTOR, SAW, or ASH2L. Two unique hairpins for SAW and ASH2L were used. In each graph, the test hairpin (red) is compared to a GFP control hairpin (black).

(H) We propose a model in which *SAW* (green) gene expression is positively controlled by mTOR signaling, while *ASH2L* transcription (red) is suppressed by mTOR signaling.

significantly increasing expression upon starvation (14 total) passed our cutoffs to be functionally analyzed, 4 of them (28%) did significantly increase expression in response to rapamycin. One of those genes encodes Thor, the *Drosophila* 4E-BP ortholog. Both the Zinke et al. study and our study used early versions of the *Drosophila* Affymetrix chip ("DrosGenome1"), and therefore there might be additional nutrient- and rapamycin-sensitive genes to be discovered.

An appreciation for the role of mTOR in tumorigenesis has emerged from clinical trials indicating that rapamycin might be an effective treatment for some cancers (reviewed in [1]). A general idea is that upregulated mTOR signaling provides tumors with a growth advantage by promoting translation initiation through S6K1 and 4E-BP1. However, it cannot be ruled out that other mTOR-regulated process such as transcription and autophagy are relevant in cancer and other diseases. These processes are conserved in yeast and represent ancient functions of TOR. Perhaps mTOR-regulated genes could be important targets for antigrowth drugs. The fact that many of the growth genes we identified block the TSC2 RNAi-induced cell size increase when cosilenced supports such a notion.

Genes with specific links to human disease were also identified in this report. *Nop60B* (CG3333) is the *Drosophila* ortholog of human *DKC1 (dyskerin),* the gene mutated in dyskeratosis congenita (DC) [48]. DC is a rare X-linked recessive disease initially characterized by nail dystrophy, abnormal skin pigmentation, mucosal leucoplakia, and premature aging, with patients often succumbing to bone marrow failure before the age of 30 [52]. DKC1 encodes a pseudouridine synthase, which associates with box H/ACA small nuclear RNAs and posttranscriptionally modifies rRNA by converting uridine to pseudouridine. In yeast, the DKC1 ortholog (Cbf5p) associates with Nhp2p [52], the *Drosophila* ortholog of which (CG5258) was also identified in our screen. Patients with DC are predisposed to tumor formation, and this is mimicked in a mouse model in which half of *Dkc1* mutant animals develop tumors [53]. It is perhaps paradoxical that mutations apparently compromising ribosome function promote tumorigenesis. However, a screen for cancer genes in zebrafish identified several ribosomal protein genes as haploinsufficient tumor suppressors [54], suggesting that ribosome dysfunction may have an important but undefined role in promoting tumor formation.

The human *ash2*/CG667 gene product (ASH2L) was discovered in a histone methyltransferase (HMT) complex with the tumor suppressor menin [55]. Menin is encoded by the *MEN1* gene, which is mutated in familial multiple endocrine neoplasia type 1 [56–58]. Several *MEN1* point mutations found in tumors are associated with reduced HMTase activity of the complex [55]. Another report found ASH2L associated with a HMT complex containing the Leukemia protooncoprotein MLL, the human ortholog of *Drosophila* trithorax, in addition to menin [59]. Interestingly, *Drosophila ash2* mutant cells in genetic mosaics exhibit defective cell differentiation and increased cell size, consistent with our conclusions [60]. In another report, *Drosophila* ASH2 localized to the nucleolus, suggesting that ASH2 might have a role in rDNA transcription [61]. The yeast ortholog of ASH2 is part of the SET1 complex, which in one study was reported to repress rDNA transcription by promoting H3 Lys4 methylation of rDNA [62, 63]. Elucidating the function of ASH2 in cell growth and differentiation might uncover clues to understanding the tumor-suppressor functions of these HMT complexes.

Conclusions

In this report we combined gene expression profiling with functional analysis by RNAi to identify *Drosophila* genes that are responsive to acute rapamycin treatment and that regulate cell growth and proliferation. This approach allowed us to functionally annotate 54 *Drosophila* genes. Most of the genes have orthologs in species ranging from yeast to mammals, and some are implicated in human disease. With genome-scale RNAi libraries becoming available in many organisms, we conclude that similar combinatorial approaches might be useful in determining subsets of "enriched" genes that could be functionally analyzed by targeted RNAi. This study found a rapamycin-sensitive growth-gene set in *Drosophila* cultured cells. In addition to emphasizing the role of TOR-dependent transcription in growth, we identified human genes with similar transcriptional and functional roles. We further conclude that similar approaches in model systems with comparatively simpler genomes can be an effective way to predict human gene function.

Experimental Procedures

Sample Preparation for Affymetrix *Drosophila* GeneChip Array analysis

Drosophila S2 cells were diluted into 6-well culture dishes at 2.5×10^6 cells/ml in a total volume of 3 mL 24 hr prior to drug addition. The following day, rapamycin was added into the culture medium to a final concentration of 20 nM. Control wells were treated with ethanol, the vehicle solvent for rapamycin. Cells were allowed to incubate for 30, 60, or 120 min before total RNA was isolated by an RNeasy kit (Qiagen). Total RNA isolation was performed in duplicate for each time point except T_0, which was done in quadruplicate. Early time points were chosen to avoid gene expression changes that might be secondary effects of arresting division. Total RNA was processed for GeneChip analysis by the Whitehead Institute center for microarray technology with the Enzo BioArray HighYield RNA transcript labeling kit. The final target mix was hybridized to *Drosophila* Affymetrix GeneChips (Affymetrix DrosGenome1) and scanned with a GeneChip Scanner 3000.

GeneChip Data Analysis

Expression values for each probe set were calculated with the Affymetrix MAS 5.0 algorithm and transformed for each chip to produce a global median of 100. Replicates were combined to calculate fold changes, and differential expression was determined with Welch's test (raw $p < 0.01$) (by means of the four 0 time point chips as controls), together with fold change (>2). Few genes showed significant differential expression on the ethanol control chips for each time point; those that did were considered ethanol-specific genes and removed from the final probe-set list. All remaining probe sets showing differential expression for at least one time point were compiled, log transformed, grouped according to up/down profiles across three time points and ordered by decreasing magnitude of change. K-means clustering was performed by means of the Pearson correlation (uncenterred) metric with Cluster 3.0. Heatmaps were produced with Java TreeView. A table of genes and their expression values, fold change, and significance values after rapamycin treatment or knockdown can be found on our website at http://web.wi.mit.edu/sabatini/pub/protocols.html. For Gene Ontology analysis, we used Flybase annotations (http://flybase.bio.indiana.edu/). Overrepresented GO terms were identified by comparison with LocusLink *D. melanogaster* genes by the hypergeometric distribution. Only 29% of the genes represented on these GeneChips had GO annotations.

RNAi Methods

A detailed description of our *Drosophila* RNAi methods can be found at http://jura.wi.mit.edu/sabatini_public/fly_array/ and is discussed in the Supplemental Experimental Procedures.

Mammalian RNAi methods are described in [8]. The sequences of the SAW and ASH2L oligonucleotides are listed in the Supplemental Experimental Procedures.

Cell Size Analysis

Cell size and number was measured with a coulter counter (Beckman Coulter). For cell size analysis after knockdown, S2 cells were incubated 4 days after dsRNA addition and then resuspended by gently pipetting up and down 10–15 times to assure even distribution of cells. A 100 μl volume of cells was subsequently added to 9.5 ml of Isoton II Diluent and mixed by inverting two times, and cell size and number were measured. Silencing events that affected cell size in our functional analysis of rapamycin-sensitive genes were repeated with newly synthesized dsRNA for confirmation. Similar methods were employed for measuring mammalian cell size except that cells were first trypsinized, then resuspended in cell culture medium. Cell-size distribution curves were generated by plotting cell size versus the number of cells in each bin of the indicated size. Testing for significant cell size change was performed with Welch's test, with p value adjustment by the FDR procedure.

Immunoblotting

A detailed description of the cell lysis methods can be found in the Supplemental Experimental Procedures. Antibodies were obtained from the following sources: phospho-T389 S6K1 and Raptor antibodies from Cell Signaling. Total *Drosophila* S6K antibody from Mary Stewart, North Dakota State University; mTOR and mammalian S6K1 antibody from Santa Cruz; and ASH2L antibody from Bethyl Laboratories.

Real-Time Quantitative RT-PCR

A master mix containing TaqMan Universal PCR Master MIX (Abi 4304437), primer/probe set, and ddH$_2$0 was prepared and distributed in a 384-well qPCR plate (Abi 4309849). GAPDH was used as the control. cDNA, prepared with a SuperScript first strand synthesis system (Invitrogen) from total cellular RNA isolated with an RNeasy kit (Qiagen), was added to the reaction mix. The reaction was run on an ABI Prism 7900HT real-time PCR machine. For each experiment, reactions were done in triplicate (n = 3), resulting in a standard deviation and error of less than 0.5 in all cases.

Supplemental Data

Supplemental Data include three figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/10/■■■/DC1/.

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