

A Role for p38 Stress-Activated Protein Kinase in Regulation of Cell Growth via TORC1^{∇†}

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The target of rapamycin (TOR) complex 1 (TORC1) signaling pathway is a critical regulator of translation and cell growth. To identify novel components of this pathway, we performed a kinome-wide RNA interference (RNAi) screen in *Drosophila melanogaster* S2 cells. RNAi targeting components of the p38 stress-activated kinase cascade prevented the cell size increase elicited by depletion of the TOR negative regulator TSC2. In mammalian and *Drosophila* tissue culture, as well as in *Drosophila* ovaries *ex vivo*, p38-activating stresses, such as H₂O₂ and anisomycin, were able to activate TORC1. This stress-induced TORC1 activation could be blocked by RNAi against mitogen-activated protein kinase 3 and 6 (MKK3/6) or by the overexpression of dominant negative Rags. Interestingly, p38 was also required for the activation of TORC1 in response to amino acids and growth factors. Genetic ablation either of *p38b* or *licorne*, its upstream kinase, resulted in small flies consisting of small cells. Mutants with mutations in *licorne* or *p38b* are nutrition sensitive; low-nutrient food accentuates the small-organism phenotypes, as well as the partial lethality of the *p38b* null allele. These data suggest that p38 is an important positive regulator of TORC1 in both mammalian and *Drosophila* systems in response to certain stresses and growth factors.

The target of rapamycin, TOR, is a highly conserved serine/threonine kinase that is a critical regulator of cell growth. It is a core component of two signaling complexes, TORC1 and TORC2 (60, 74). TORC1 is defined by the presence of Raptor in the complex, while TORC2 contains Rictor. Rictor and Raptor are mutually exclusive. Activation of the TORC1 pathway leads to increased protein translation, increased cell size, and increased proliferation, making this pathway an important target for emerging cancer therapies. Rapamycin is an inhibitor of TORC1 that is commonly used as an immunosuppressant following kidney transplantation (51). At least three analogs of rapamycin are currently being tested in solid and hematological tumors and have shown some promising results (21).

The TORC1 pathway responds to numerous inputs, sensing both the desirability of and the capacity for growth. Many of these pathways control TORC1 signaling through phosphorylation of the tuberous sclerosis protein TSC2. TSC2 associates with TSC1 to form a heterodimeric GTPase-activating protein complex (GAP) that inactivates the small GTPase Rheb (24, 29, 67). While the exact molecular mechanism remains a topic of debate, activation of Rheb promotes the kinase activity of TORC1 (24, 29, 67). Rheb is required for the activation of TORC1 in response to both amino acids and growth factors

(55, 62). In *Drosophila melanogaster*, mutation of either TOR or Rheb inhibits growth, leading to reduced body size and reduced cell size in mutant clones (42, 64). Mutation of either TSC1 or TSC2 has the predicted opposite effect, as tissue deficient for either of these proteins overgrows and contains large cells (49, 66).

TORC1 is activated via the phosphatidylinositol 3' kinase (PI3'K) pathway by growth-promoting mitogens, such as insulin and growth factors. *Drosophila* mutants with mutations of PI3'K pathway components have size phenotypes similar to those of the TOR and Rheb mutants (71). In mammalian cells, the PI3'K-mediated activation of TORC1 occurs at least in part through the phosphorylation of TSC2 by the PI3'K target AKT (30, 50). Interestingly, mutation of these residues in *Drosophila* has no impact on TSC2 function *in vivo*, suggesting that there may be other mechanisms through which PI3'K can activate *Drosophila* TOR (20). Recent work has suggested that the proline-rich AKT substrate PRAS40 may provide part of this link (23, 59, 69, 70). In addition, signaling through RAS activates extracellular signal-regulated kinase (ERK) and ribosomal S6 kinase (RSK), which can phosphorylate TSC2 and Raptor to activate TORC1 (13, 40, 56). There are also likely to be additional mechanisms through which growth factors activate *Drosophila* TOR that have not yet been identified.

TORC1 activity is also controlled by the intracellular building blocks necessary to support cellular growth. The energy-sensing AMP-activated protein kinase (AMPK) pathway relays information about the energy status of the cell to TORC1 by phosphorylating TSC2. Unlike the inactivating phosphorylation of TSC2 by Akt, phosphorylation of TSC2 by AMPK

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promotes the GAP activity of the TSC complex (31). AMPK also phosphorylates Raptor, leading to decreased TORC1 activity (28). Thus, when energy levels are low, active AMPK inhibits TORC1.

Amino acids also activate the TORC1 pathway, through a mechanism that requires Rheb, as well as the type III PI3'K VPS34 and the serine/threonine kinase mitogen-activated protein kinase kinase kinase 3 (MAP4K3) (11, 22, 43). TORC1 thereby integrates information about the availability of amino acids and the amount of energy available for growth with growth factor signaling. Given its ancient function in adapting growth rates to environmental conditions, it is likely that TOR responds to a variety of stimuli, suggesting that many TOR control mechanisms remain to be uncovered. The Rag family of Ras-related small GTPases has recently been identified as a key component of the amino acid-sensing pathway, acting in parallel to Rheb (34, 58). Rag GTPases form heterodimers; RagA or RagB interacts with RagC or RagD. RagA and RagB are active when GTP bound, while RagC and RagD are active when bound to GDP (34, 58). Activation of the Rags by amino acids results in TOR relocation to Rab7-containing vesicles (58). While the function of these vesicles in TORC1 signaling remains unclear, this relocation is associated with increased TORC1 activity.

TORC1 controls cell growth and translation through the phosphorylation and activation of components of the translational machinery, such as S6 kinase (S6K) and 4EBP1, an inhibitor of eukaryotic translation initiation factor 4E (eIF4E) activity (reviewed in reference 27). S6K phosphorylates the S6 ribosomal subunit, thereby increasing translation. Mice deficient for S6K1 are small and have small pancreatic beta cells and a correspondingly low level of circulating insulin (45). Mutation of the phosphorylation sites on S6 results in a similar phenotype, with small beta cells and fibroblasts (57). In *Drosophila*, mutation of S6K again reduces both cell and organism size (42), as does the overexpression of 4EBP (41).

Interestingly, while mutation of the TORC1 pathway in mammalian cells reduces cell size by 10 to 15%, ablation of core TORC1 pathway components in *Drosophila* cells can affect cell size by up to 40% (73). In an attempt to identify novel components of the TORC1 pathway, we undertook an RNA interference (RNAi)-based screen of *Drosophila* S2 cells. We reasoned that the extreme size phenotypes observed in *Drosophila* cells upon TORC1 manipulations would facilitate the identification of modulators. In order to increase the likelihood of isolating novel regulators of TOR, we uncoupled TOR activity from many of its known nutritional controls by depleting TSC2 and screened for double-stranded RNAs (dsRNAs) that could reverse the cell size increase elicited by loss of TSC2. Depletion of multiple components of the p38 pathway was found to revert the TSC2 RNAi-induced cell size increase. Furthermore, activation of p38 is necessary and sufficient for the activation of TOR. Strikingly, mutation of components of the stress-activated p38 pathway in *Drosophila* has a similar phenotype to mutations in the TOR and insulin signaling pathway: a cell-autonomous cell size decrease, reduced body size, and a sensitization to the effects of nutritional stress.

MATERIALS AND METHODS

Chemicals. Anisomycin, rapamycin, SB202190, insulinlike growth factor (IGF), epidermal growth factor (EGF), and 4-hydroxytamoxifen (4-OHT) were from Calbiochem. H₂O₂ was from BDH Laboratories, and bovine insulin was from Sigma-Aldrich.

RNAi screen. An RNAi library targeting 335 kinases and phosphatases was generated. Primer information can be found at <http://flight.licr.org>. S2 cells were plated in 96-well plates, and the library RNAi was transfected along with Tsc2 RNAi using Fugene 6. After five days, cells were harvested, diluted in phosphate-buffered saline (PBS), and analyzed using a Z2 Coulter counter (Multisizer II; Beckman-Coulter).

Primer sequences for dsRNA. Primer sequences for dsRNAs are as follows: for TOR, TAATACGACTACTATAGGCTTTTGAGGTGCTCAGAGGC and TAATACGACTACTATAGGGTAGCCGCGGACTAGAGTAT; for S6K, TAATACGACTACTATAGGTCAGTTGAGGTGCTGTTTGA and TAATA CGACTACTATAGGGCGTGAGGGCATTCTTAG; for TSC1, TAAT ACGACTACTATAGGGGTGAGGATGCTGACAGCCTTCTC and TAATA CGACTACTATAGGGTATGTCAGTTCTGTCCGTGCTCC; for TSC2, TAAT ACGACTACTATAGGAATGTGCTGACAGCCTTCTC and TAATACGAC TCACTATAGGGGACACTCGACTCCAGATGA; for 4EBP, TAATACGA CTCACTATAGGGGAATCAGTAAAGTGGCCTC and TAATACGACT CACTATAGGGACAAGGTAACGGGGTCAATATG; for Lic, TAATACGA CTCACTATAGGGTAAGCAAACCGATACGGTCC and TAATACGACT ACTATAGGGTACCAGCCAGAGGTAGGCAC for set 1 and TAATACGAC TCACTATAGGGGAGTATAGGCAAGGCCAA and TAATACGACTCAC TATAGGGTGACGCTTATTGCTTATTGCTGATTGC for set 2; for Mekk1, TAATACGACTACTATAGGGCGTTCGAAAAGGTACTTG and TAAT ACGACTACTATAGGGTGTCAACGATGAGAGCAAGC for set 1 and TA ATACGACTACTATAGGGTACCAATGGCTCGACT and TAATAC GACTACTATAGGGTGACAGGCACTCTACCTT for set 2; for MK2, TAATACGACTACTATAGGGGATTGCCAGTACAATGCT and TAA TACGACTACTATAGGGATCTACGCCATCTCTCTCCT; for Gcn2, TAA TACGACTACTATAGGACGAGTGCCTACTGTGCATC and TAATACG ACTACTATAGGGGCTGGACGGTGTAGGATGT; for MAST205, TAA TACGACTACTATAGGGCAACTCAAACCTCAGGCGACA and TAATAC GACTACTATAGGGTGATGCTTACACTGCTTCC; for Trc, TAATACG ACTACTATAGGGGTAGCAGCTTGAAGGTGCC and TAATACGACT CACTATAGGGGAACCAATGGAAGGACATTTG; for IKKb, TAATACGAC TCACTATAGGGTGCAGCTGTATGTGGAGGAG and TAATACGACTCA CTATAGGGTCTCGAAACTTCTTCCGT; for Psk, TAATACGACTCACT ATAGGGCCTGTCCGACTCTTTTGAGC and TAATACGACTACTATAG GGAAGCGGTGTATCTGGTTTGG; for Pdp1, TAATACGACTACTATAG GGTGCGAAAAACCCCTTCATC and TAATACGACTACTATAGGGT GGTTTTGACATTTTCCA; for Puc, TAATACGACTCACTATAGGGGAG TGTGCGTGTACAAGTGG and TAATACGACTCACTATAGGGCGCTT TATCCGCAATTTTCAT; for p38a, TAATACGACTCACTATAGGGCCCTT CTAAGCAATACCA and TAATACGACTCACTATAGGGTCAAGTCTG CGTCCATCAAG; and for p38b, TAATACGACTCACTATAGGGGACCT GAGCGTACAGAACA and TAATACGACTCACTATAGGGGACCTGAG CGTACAGAACA.

Semiquantitative reverse transcription-PCR (RT-PCR). RNA was prepared from S2 cells using a Qiagen RNeasy mini kit. One microgram of total RNA was used for cDNA synthesis using an Advantage RT-for-PCR kit (Clontech). The same gene-specific primers listed above were used for amplification from total cDNA, except for Licorne, for which the primers GAACAGCACCCCTTG TAAT and GTGACGCTTATTGCTGATTGC were used. GGACGATATGG AGAAGATCTGG and CATGATCTGGTTCATCTTCTCA were used to amplify actin.

Transfection of RNAi oligonucleotides into *Drosophila* S2 cells. RNAi molecules were generated as described previously (73). S2 cells were plated in serum-free medium and incubated with dsRNA. Serum-containing medium was added after 1 h. Cells were harvested after five days. For primer information, see materials and methods in the supplemental material.

Transfection of RNAi oligonucleotides into human cell lines. A pool of four (SMART pools; Dharmacon) pre-designed small interfering RNA (siRNA) molecules were used for all transfections except for MKK3/6, where Ambion siRNA against MKK3 and MKK6 were pooled together. Cells were transfected using Dharmafect 1 and assayed after three days.

Western blotting. Cells were harvested directly into protein sample buffer (Invitrogen). Western blots were blocked in TBS-T (Tris-buffered saline containing 0.1% Tween-20) with 5% milk, probed with primary antibody, washed thrice with TBS-T, incubated with horseradish peroxidase (HRP)-conjugated

secondary antibodies, and washed again thrice in TBS-T. Antibodies against phospho-S6, phospho-4EBP1, S6, 4EBP1, TSC2, p38, mammalian TOR (mTOR), phospho-S6K, MKK3, MKK6 (Cell Signaling Technology), actin, mTOR (Santa Cruz), phospho-p38 (Biosource), Raptor (Novus), *Drosophila* S6K (gift from D. Alessi), polyglutamate (EE), hemagglutinin (HA) (CRUK monoclonal antibody production facility), phospho-Raptor S863 (Abgent), tubulin, and armadillo (Developmental Studies Hybridoma Bank) were used.

Constructs. A cDNA library generated from S2 cells (Advantage RT-PCR; Clontech) was used to amplify *Drosophila* S6K cDNA, using the primers AAG CTTGGTGGTGGTAACACACACACGGCAATGGCGGAC and ACTAGTA GCAATCGCTCCAGCCTTTAGACC, and cloned into pcDNA3.1Flag using the HindIII and SpeI restriction cut sites in the primers. The resulting N-terminally Flag-tagged version of S6K was excised and cloned into pMK33. Mutagenesis of T238 and T398 was performed using a QuikChange site-directed mutagenesis kit (Stratagene). Rag constructs were a gift from D. Sabatini (Addgene plasmids 19302, 19303, 19305, and 19306). To generate the Raptor S863G mutant, HA-Raptor (Addgene plasmid 8513) was mutagenized on S863 using a QuikChange XL site-directed mutagenesis kit and primers CGGCCCC CGCCGCCCCACCAAC and GTTGGTGGGGCCGGCGGGGGCCG.

Ex vivo stimulation of ovaries. Ovaries from adult females were dissected in PBS and placed into glass dishes containing Schneider's medium (Invitrogen). Ovaries were stimulated as indicated in the legend to Fig. 3, with gentle agitation for 1 h, harvested by centrifugation, lysed in protein sample buffer, and sonicated.

Stress assays. To assay high-salt stress, L1 larvae from homozygous matings were placed on medium containing 0.2 M NaCl₂ at a concentration of 25 larvae per vial. Adults were counted 12 days later. For the stress assay with 1% H₂O₂, adult flies (2 to 5 days old) were placed into vials containing 1% H₂O₂. Surviving adults were counted twice per day. For the dry starvation stress assay, adult flies (2 to 5 days old) were placed into empty vials. Surviving adults were counted twice per day. To assay heat shock stress, adult flies (2 to 5 days old) were placed into vials containing moist paper towel. Surviving adults were counted every hour.

Rheb-GTP loading. Transfected green fluorescent protein (GFP)-Rheb was immunoprecipitated from 293 cells overexpressing MEK kinase 3 (MEKK3)-estrogen receptor (ER) using 1 mg of anti-GFP antibody (3E1). Lysis, immunoprecipitation, and thin-layer chromatography were performed as previously described (39).

TAP. pMSCV-TAP Raptor (Addgene) was transfected into 293 cells, and stable clones were isolated by puromycin selection. Tandem affinity purification (TAP) was performed as described previously (www.embl-heidelberg.de/ExternalInfo/seraphin/TAP.html) except that 0.3% CHAPS {3-[3-cholamidopropyl]-dimethylammonio}-1-propanesulfonate} was used instead of 0.1% NP-40 in all of the buffers.

Immunoprecipitation. 293 cells transfected with myc-mTOR, HA-Raptor (Addgene plasmids 1861 and 8513), or GFP-Rheb (9) were lysed 72 h after transfection in CHAPS buffer (9). Immunoprecipitations were performed from 6-well dishes with 1 mg of 12CA5 (HA) or 9E10 (myc) monoclonal antibodies for 2 h and then with immunoglobulin G (IgG) for an additional hour before washing three times in CHAPS buffer.

Larval protein concentration measurements. Ten larvae per genotype were collected 72 h after egg lay and frozen in liquid nitrogen. Larvae were thawed into 0.3% CHAPS buffer, homogenized, and sonicated. Protein concentration was determined by Bradford assay (Pierce) in triplicate.

Measuring wing area and adult mass. L1 larvae were placed onto low-nutrient medium (see below) at a concentration of 50 larvae per vial. The resulting adults were weighed in groups of 20. These adults were then dehydrated in 95% ethanol, and wings were mounted for determination of area as described previously (44).

***Drosophila* media.** Each liter of high-nutrient medium contained 7.2 g agar, 36 g maize, 7.3 g yeast, 4.4 g soya, 72 g malt, 24 ml molasses, 5.2 ml propionic acid, and 74 μ l of orthophosphoric acid. Each liter of low-nutrient medium contained 10 g agar, 83 g maize, 17 g yeast, and 60 g sucrose.

Survival of *lic* null larvae. *lic*/FM7, *Kr*>GFP females were mated with FM7, *Kr*>GFP/Y; *dTor*^{2L19}/CyO, *actin*>GFP or FM7, *Kr*>GFP/Y; *dRheb*^{2D1} males to generate larvae null for both *lic* and either *dTor* or *dRheb*, respectively. *lic*/FM7, *Kr*>GFP; *da*>*Gal4*/*da*>*Gal4* females were mated with FM7, *Kr*>GFP/Y; *UAS-dRheb*⁴⁷⁻⁴⁹/*UAS-dRheb*⁴⁷⁻⁴⁹ males to generate larvae null for *lic* and overexpressing *dRheb*.

Flow cytometry analysis of wing discs. Flies of genotype *FRT19A,lic*/*FRT19A,Ub*>GFP; *hs*>*FLP* or *hs*>*FLP*; *FRP40A,p38b*/*FRT40A,Ub*>GFP; *p38a/p38a* were heat shocked at 37°C for 60 min 48 h after egg lay. Wing discs were processed as previously described (66).

RT-PCR for *lic*, *p38b*, and *actin*. Ten larvae of the indicated genotypes were collected 72 h after egg lay and frozen in liquid nitrogen. RNA was prepared using an RNeasy minikit (Qiagen), and cDNA was prepared using an Advantage RT-for-PCR kit (Clontech). The following primers were used for the PCR: for *lic*, GAACAGCACCGCCTTGTAAT and GTGACGCTTATTGCTGATTGC; for *p38b*, GCTGGAGAAGATGCTGGAAC and GCAAATCGAAGGTTCCA AAA; and for *actin*, GGACGATATGGAGAAGATCTGG and CATGATCTG GTGCATCTTCTCA.

RESULTS

An RNAi screen for modulation of TSC2-induced cell size increase. Activation of the insulin/TOR pathway in *Drosophila* S2 cells has dramatic effects on cell size (73). Treatment of these cells with dsRNAs directed against the tuberous sclerosis protein TSC1 or TSC2 increases cell size by up to 40%, while RNAi against TOR or S6K decreases cell size by approximately 20% (Fig. 1A). Once activated, TOR phosphorylates S6K and 4EBP1 (27). The phosphorylation of S6K is activating, while the phosphorylation of 4EBP1 prevents it from binding to eIF4E. Since the interaction between 4EBP1 and eIF4E is inhibitory, the phosphorylation of both S6K and 4EBP1 activates translation. Accordingly, RNAi against S6K itself results in a 20% decrease in cell size, while RNAi against 4EBP1 increases cell size (Fig. 1A). Thus, the changes in *Drosophila* S2 cell size in response to RNAi against known TOR pathway components are consistent with previously published data establishing the roles of S6K and 4EBP1 in the TOR-mediated control of cell size.

In mammalian cells, TSC1 and TSC2 have been implicated in pathways other than TOR (4, 72). In S2 cells, however, RNAi against either TOR or S6K completely reversed the large-cell phenotype induced by TSC2 RNAi (Fig. 1B). TSC2 depletion may therefore provide a useful background to identify novel components of the TOR signaling pathway, as RNAi directed against these components should reverse the large-cell phenotype induced by TSC2 RNAi. An RNAi library targeting 335 known *Drosophila* kinases and phosphatases was used to search for novel TOR pathway components. In this screen, cells were treated with RNAi against TSC2 in combination with individual dsRNAs from the library, and cell size and cell number were measured using a Coulter counter (Fig. 1C and D).

Of the 5% of dsRNAs that reduced cell size the most dramatically, three were known components of the TOR signaling pathway (Fig. 1C and D): TOR itself, S6K, and phosphoinositide-dependent kinase 1 (PDK1), a kinase known to be required for S6K activation (5, 36, 54). RNAi against any of these three kinases was able to reverse the TSC2-mediated increase in cell size (Fig. 1C; also see Table S1 in the supplemental material). Other RNAi molecules within this top 5% of "hits" include I κ B kinase β (IKK β), which has recently been shown to activate TOR (37); two regulators of apoptosis (DIAP1 and Wts); and other kinases, such as CG14163, MYT1, and MAST205 (see Table S1 in the supplemental material).

Interestingly, among the 5% of dsRNAs with the strongest effect in reversing the large-cell phenotype were three core components of the stress-activated p38 signaling pathway: Licorne, Mekk1, and MAPK-activated protein kinase 2 (MK2) (Fig. 1C and D). Mekk1 phosphorylates and activates Licorne, which in turn phosphorylates and activates p38. p38 has nu-

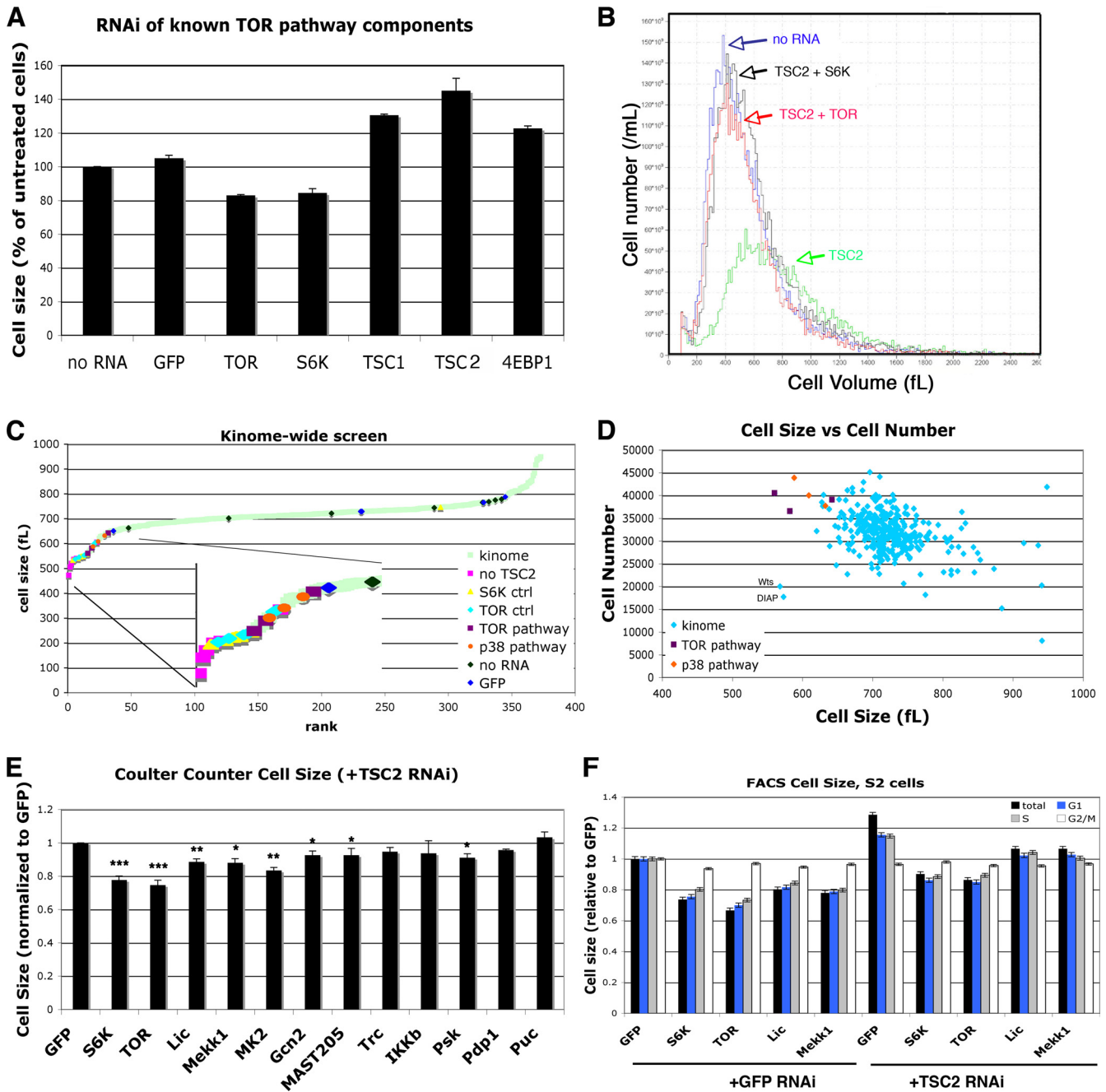


FIG. 1. A genetic screen for regulators of TSC2-mediated cell size identifies members of the p38 pathway. (A) RNAi targeting known members of the Tor pathway in *Drosophila* S2 cells. S2 cells were treated with the indicated RNAi for 5 days, and cell size was measured by Coulter counter. Error bars represent standard errors of the means (SEMs) across 3 independent experiments. (B) RNAi targeting either S6K or TOR can rescue the large-cell phenotype caused by RNAi targeting TSC2. Cells were treated with both RNAis together for 5 days. Cell size was measured using a Coulter counter. (C, D) S2 cells were treated with TSC2 RNAi together with RNAi molecules targeting each *Drosophila* kinase or phosphatase. “TOR ctrl,” “S6K ctrl,” and “GFP” indicate RNAi molecules that were synthesized independently from the rest of the RNAi collection. “TOR pathway” and “p38 pathway” indicate RNAi molecules targeting components of these two pathways within the screened RNAi collection. (C) Each RNAi in the screen is presented in order of increasing cell size. (D) Components of the p38 pathway affect both cell size and cell number. RNAi molecules targeting components of either the TOR or the p38 pathway result in increased cell number and decreased cell size. Data points in panels C and D are averages of two independent repeats. (E) A second, nonoverlapping RNAi was used to validate 10 RNAi molecules identified in the screen. As in the initial screen, S2 cells were treated with the indicated RNAi for 5 days, either alone or with TSC2 RNAi. Error bars represent SEMs across 3 independent experiments. (F) Cells were treated with RNAi as indicated, together with either TSC2 or GFP RNAi. Cells were harvested, stained with propidium iodide, and analyzed by flow cytometry. All populations were normalized to the results for GFP RNAi, set to 1, to allow for comparisons between experiments. The forward scatter of the total population, as well as of the G₁-, S-, and G₂/M-gated populations, are shown. Error bars represent the SEMs within one representative experiment. ***, $P < 0.005$; **, $P < 0.01$; *, $P < 0.05$.

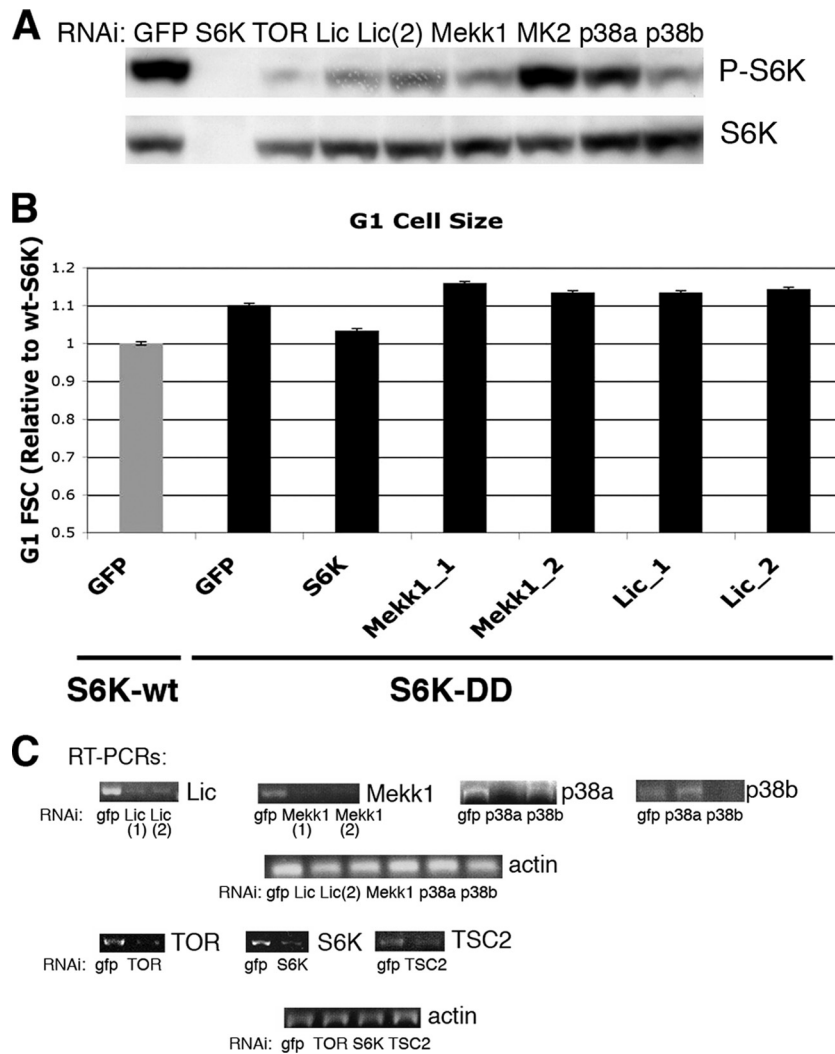


FIG. 2. Licorne RNAi prevents the cell size increase mediated by Tsc2 RNAi but does not affect the size of cells overexpressing S6K-DD. (A) S2 cells were treated with Tsc2 RNAi plus the indicated RNAi. Total cell extracts were immunoblotted with the indicated antibodies. (B) S2 cells were stably transfected with either wild-type S6K (S6K-wt) or with a mutated form of S6K in which both T238 and T398 were mutated to aspartate (S6K-DD). Pools of stably transfected cells were treated with RNAi as indicated, stained with propidium iodide, and analyzed by flow cytometry. The forward scatter (FSC) for the G₁-gated population is shown. Error bars represent the SEMs from a representative experiment. (C) Semiquantitative RT-PCR was used to measure the indicated mRNA species in S2 cells treated with RNAi for 5 days.

merous downstream targets, including MK2. In mammals, MK2 may also be involved in a positive feedback loop, as RNAi against MK2 destabilizes p38 (35). Neither of the *Drosophila* p38 homologues p38a and p38b were included in the library, and they were therefore not recovered in our screen.

In addition to altering cell size, RNAi against TSC2 also affects cell proliferation (6) (Fig. 1D). Rapamycin, a TOR inhibitor, inhibits progression from G₁ to S phase and induces a G₁ arrest in many mammalian cells. However, rapamycin treatment and TOR inhibition also accelerate the progression from G₂ into M (47, 73). In S2 cells, the latter mechanism predominates, and inhibition of the TOR pathway with either low levels of rapamycin or RNAi against the insulin/TOR pathway accelerates progression through G₂/M and increases cell number (73). Consistent with these observations, treatment of S2 cells with TSC2 RNAi decreases cell number, and the TSC2 RNAi-mediated decrease in

cell number can be reversed by RNAi against S6K, TOR, Mekk1, Lic, or MK2 (Fig. 1D). In contrast, RNAi against Wts or DIAP1, while reducing cell size, also reduced cell number (Fig. 1D). Given their known roles in inhibiting apoptosis, Wts and DIAP1 were excluded from further analysis.

To confirm some of the results of this screen, second, non-overlapping RNAis targeting eight genes identified as putative negative regulators, as well as two genes (Puc and Pdp1) identified as putative positive regulators, were generated. S2 cells were treated with these RNAis with TSC2 RNAi, and cell size was measured by Coulter counter. Targeting of any of the three identified p38 pathway components with this second RNAi also decreased the size of cells treated with TSC2 RNAi. The levels of mRNA remaining after selected RNAi treatments in S2 cells are shown in Fig. 2C.

As cells progress through the cell cycle, they grow before

they divide. Thus, RNAis that block cells in G₂/M would be predicted to increase the average cell size of a population due to an accumulation of the larger G₂ cells. Conversely, RNAis that block cells in G₁/S should decrease the average size. Indeed, many of the RNAi molecules that increased cell size in our screen are known cell cycle regulators (see Table S1 in the supplemental material). p38 β has been identified as a regulator of cell cycle (and therefore cell size) in a genome-wide RNAi screen (6). To distinguish *bona fide* regulation of growth from changes in cell cycle progression, cells were treated with RNAi and subjected to fluorescence-activated cell sorting (FACS) analysis. By analyzing forward scatter and, hence, cell size, we could demonstrate that both TOR and p38 pathway components reduced cell size in all phases of the cell cycle (Fig. 1F). Interestingly, treatment of cells with RNAi targeting p38 pathway components alone also had a small effect on cell size (Fig. 1F). Thus, both TOR and p38 pathway components affect both cell size and cell cycle, but the cell size effect is likely due to altered cell growth rather than the consequence of a cell cycle phasing defect.

Inhibition of p38 signaling decreases phosphorylation of S6K. We next sought to investigate the molecular interaction between p38 and TORC1 signaling. First, we examined whether inhibition of p38 signaling had any effect on the phosphorylation of the TOR target S6K. As S6K phosphorylated at T398 (P-S6K) is not detectable in unstimulated S2 cells, TSC2 RNAi was used to activate TOR and induce S6K phosphorylation. RNAi against Lic, Mekk1, and p38b dramatically reduces the TSC2 RNAi-mediated phosphorylation of S6K (Fig. 2A). In contrast, p38a and MK2 RNAi had little effect. This may be due to inefficient RNAi, or MK2 may affect cell size through another mechanism.

To investigate this possibility further, we generated S2 cells stably expressing S6K in which two activating phosphorylation sites, T238 and T398, were mutated to phosphomimetic sites (S6K-DD) (10, 52). Mutation of these two sites constitutively activates S6K and increases cell size (Fig. 2B). In these cells, RNAi against p38 pathway components does not affect cell size (Fig. 2B). Thus, S6K activation is dominant to p38, and activation of p38 results in phosphorylation of S6K. Taken together, these results suggest that p38 acts upstream of S6K in the control of cell growth.

Activation of p38 results in the phosphorylation of TOR targets. p38 becomes activated in response to numerous stresses (1, 16, 76). This occurs through the phosphorylation of both the Thr and Tyr within the primary sequence TGY of p38 by the upstream kinase Lic (*Drosophila*) (MKK3 and MKK6 in mammals). Whereas previous work has suggested that TOR becomes inhibited upon the induction of stresses such as hypoxia (3, 17, 53, 63), our data demonstrate that RNAi against p38 prevents phosphorylation of S6K, suggesting that in some situations stress-induced activation of p38 may play a positive role in the activation of TOR targets (Fig. 2A). S2 cells were therefore treated with one of the stress-inducing reagents H₂O₂ and anisomycin, and levels of phosphorylated S6K were measured (Fig. 3A). Treatment of cells with anisomycin for 1 h increased both phosphorylation of the TGY motif of p38 (P-p38) and phosphorylation of S6K.

Drosophila ovaries are sensitive to manipulation of the insulin/TOR pathway (44). Furthermore, the p38 pathway is

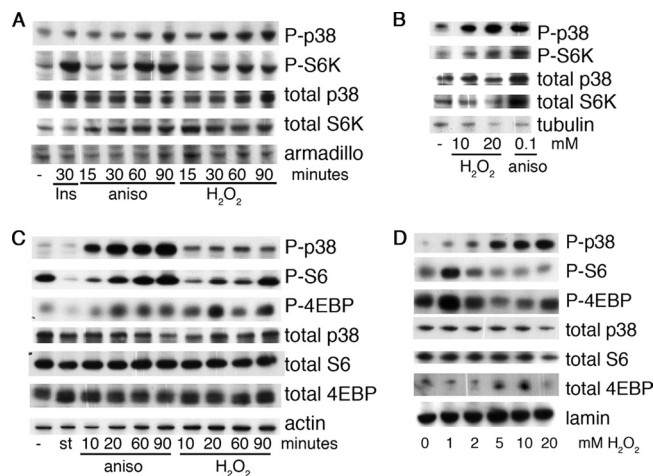


FIG. 3. Stimulus and dose-specific activation of p38 induces phosphorylation of Tor targets. (A) S2 cells were treated with 1 μ M insulin (Ins), 10 μ g/ml anisomycin (aniso), or 1 mM H₂O₂ as indicated. Cell extracts were collected and analyzed by immunoblotting. (B) Ovaries from *w¹¹¹⁸* females were dissected into PBS and stimulated for 1 h at room temperature as indicated. The ovaries were lysed in protein sample buffer, sonicated, and analyzed by immunoblotting. (C) A549 cells were starved of serum overnight and then starved of amino acids for 90 min (st), treated with 10 μ g/ml anisomycin (aniso) or 1 mM H₂O₂ for the indicated times, and analyzed by immunoblotting. —, cells were grown in 10% serum. (D) Induction of S6 phosphorylation by H₂O₂ is dose dependent. A549 cells were treated for 30 min with the indicated concentrations of H₂O₂ and analyzed by immunoblotting.

important in the developing ovary, as deletion of either the upstream p38 kinase *lic* or p38b itself results in defects in oogenesis (65). Interestingly, *ex vivo* stimulation of ovaries with anisomycin results in the phosphorylation of both p38 and S6K (Fig. 3B).

We wished to see if the effects of p38 on TORC1 were conserved in mammals. Similar to the results seen in *Drosophila* S2 cells, stimulation of human A549 cells with either anisomycin or H₂O₂ increased both phospho-p38 and phospho-S6 (S235/236) (P-S6) (Fig. 3C). Interestingly, the induction of S6 phosphorylation in A549 cells was dependent upon the concentration of H₂O₂ used (Fig. 3D). Phosphorylation of S6 was seen only with the lower doses of H₂O₂; higher doses of H₂O₂ did not induce the phosphorylation of S6 (Fig. 3D). A similar pattern of phosphorylation was observed in another TORC1 target, 4EBP (T37/46) (P-4EBP), suggesting that p38 acts upstream of TORC1 (Fig. 3C and D). Thus, activation of p38 in *Drosophila* cell lines, human cell lines, and *Drosophila* ovaries results in the phosphorylation of S6K and its downstream target S6, confirming that p38 signaling likely acts upstream of S6K phosphorylation in the TOR pathway.

Activation of p38 increases cell size in human cells. Most of the core components of the TOR signaling pathway are well conserved between humans and *Drosophila*. To examine whether the p38 pathway can also affect cell size in mammalian cells, we treated A549 cells with RNAi against TSC2 or against the mammalian homologues of Licorne, MKK3 and MKK6. Similar to the results obtained in *Drosophila* S2 cells, RNAi against MKK3 and MKK6 could prevent the cell size increase induced by TSC2 RNAi (Fig. 4A). The levels of TSC2 protein remaining after siRNA treatment are shown (Fig. 4B). The

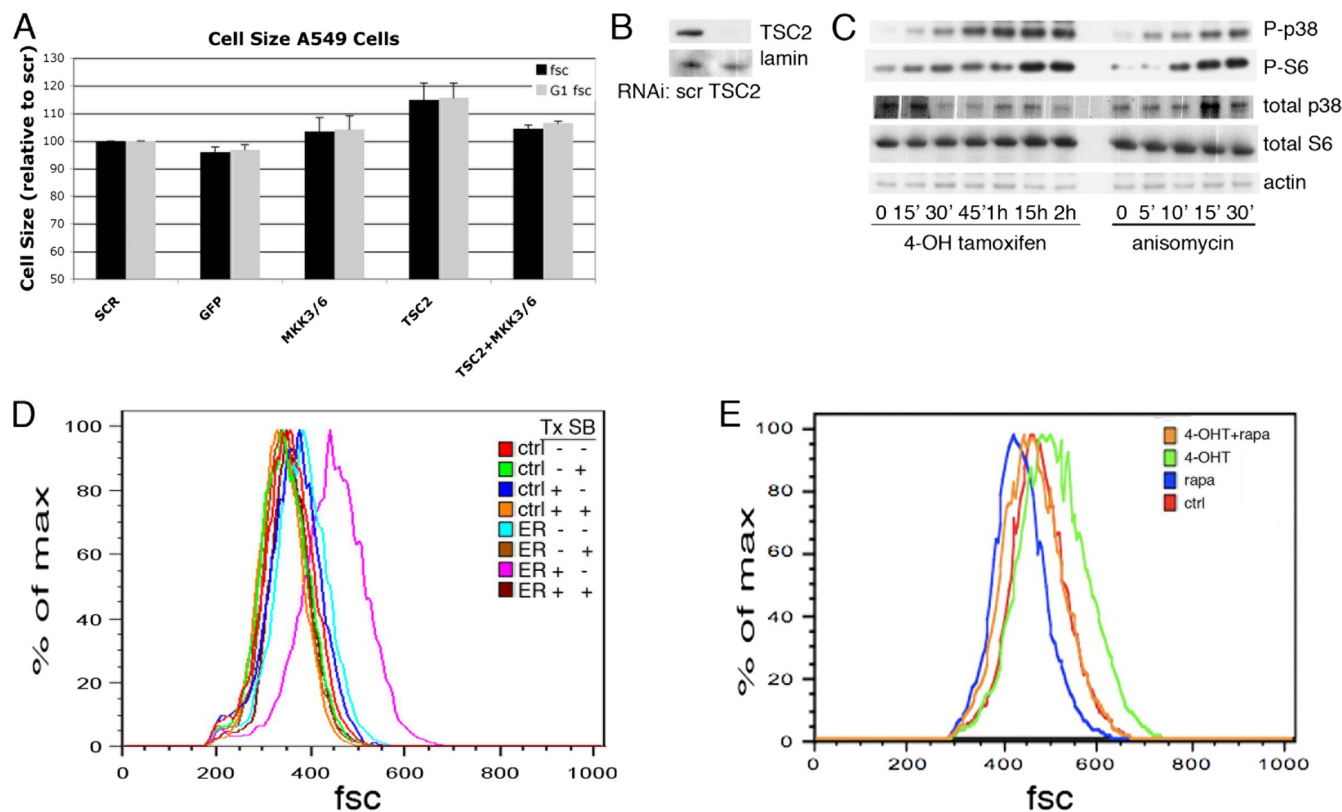


FIG. 4. Activation and inhibition of the p38 pathway alter cell size. (A) A549 cells were treated with the indicated RNAi for 3 days, stained with propidium iodide, and analyzed by flow cytometry. The forward scatter (fsc) of the total population, as well as of the G_1 -gated population, is shown. Error bars represent SEMs of this representative experiment. (B) Western blot analysis indicating the amount of TSC2 protein remaining following a three-day treatment with TSC2 siRNA. (C) Phosphorylation of p38 and S6 is induced by 4-hydroxytamoxifen treatment of 293 cells stably transfected with MEKK3-ER. 293 cells stably expressing MEKK3-ER were treated with 100 nM 4-hydroxytamoxifen or 10 μ g/ml of anisomycin for the indicated amount of time (' , minutes). Total cell lysates were analyzed by immunoblotting with the indicated antibodies. (D, E) Long-term activation of MEKK3-ER by 4-hydroxytamoxifen (4-OHT) treatment increases cell size. (D) Control 293 cells (ctrl) or 293 cells stably expressing MEKK3-ER (ER) were treated with 4-hydroxytamoxifen (Tx) and/or SB202190 (SB) for 24 h as indicated, stained with propidium iodide, and analyzed by flow cytometry. The results for the G_1 -gated population are shown. (E) 293 cells stably expressing MEKK3-ER were treated with 4-hydroxytamoxifen and/or rapamycin (rapa) as indicated, stained, and analyzed as described for panel D. scr, scrambled siRNA control.

MEKK3-ER fusion protein contains the kinase domain of MEKK3, the *Drosophila* Mekk1 homolog and an upstream activator of p38, fused to a modified form of the tamoxifen-responsive ER domain of the estrogen receptor (25). Treatment of these cells with 4-hydroxytamoxifen (4-OHT) activates the p38 pathway and, consistent with the results presented in Fig. 3, induces the phosphorylation of S6 (Fig. 4C) (25, 68). Importantly, treatment of these cells with 4-OHT for 24 h also increased cell size in a p38-dependent manner (Fig. 4D). The tamoxifen-induced cell size increase was dependent upon TOR, as concurrent treatment with rapamycin abolished this effect (Fig. 4E).

Stresses promote S6 phosphorylation via the TOR pathway.

The TOR pathway responds to external stimuli in the form of growth factors and insulin and to internal stimuli, such as the availability of amino acids. Core components of the TOR pathway, including Rheb, TOR, and S6K, are required for the phosphorylation of S6 in response to all of these stimuli. The pathway through which stresses induce S6 phosphorylation was therefore investigated using RNAi against Rheb and TOR. Consistent with a role for p38 in the activation of the TOR pathway, RNAi against Rheb or TOR was able to prevent the

phosphorylation of S6 in response to anisomycin (Fig. 5A). Similarly, treatment of cells with the TOR inhibitor rapamycin was also able to prevent the phosphorylation of S6 in response to anisomycin (Fig. 5C).

Interestingly, RNAi against both MKK3 and MKK6 (MKK3/6) was able to prevent the phosphorylation of S6 and 4EBP in response to amino acids (Fig. 5A), insulin, or EGF (Fig. 5B). Consistent with this observation, treatment of cells with the p38 inhibitor SB202190 or BIRB 796 was able to abrogate the phosphorylation of S6 and 4EBP in response to amino acids (Fig. 5C and D). Phosphorylated p38 was not detectable in cells treated with insulin, EGF, or amino acids, suggesting that these stimuli do not directly activate p38 but, rather, that they require basal p38 activity in order to induce the phosphorylation of TORC1 targets. This suggests a role for basal p38 activity in the activation of translation in response to known TOR-activating stimuli, such as growth factors and amino acids. Thus, TOR and Rheb are required for the anisomycin-induced phosphorylation of S6 and 4EBP, and MKK3/6 is required for the phosphorylation of S6 and 4EBP in response to amino acids and growth factors.

Rags are dominant to p38 in the activation of TORC1. Rags are small GTPases recently described to activate TORC1 in response to amino acids (34, 58). In order to further characterize the level at which p38 is able to influence TORC1 activity, we activated the p38 pathway in the presence of dominant negative Rags (58). In accordance with previous data, we found that Rags are essential for S6K phosphorylation in response to amino acids (Fig. 5E). Similarly, dominant negative Rags are able to prevent the phosphorylation of S6K in response to anisomycin treatment (Fig. 5E). Constitutively activated forms of RagB and RagC can induce the phosphorylation of TORC1 targets even in the absence of amino acids (34, 58). Again, we find that constitutively activated Rags can induce the phosphorylation of TORC1 targets in the presence of siRNA targeting MKK3/6 (Fig. 5G). Taken together, these data suggest that the activation of TORC1 in response to p38 occurs upstream of Rag activation.

We investigated a number of potential mechanisms through which p38 might activate TORC1 downstream of TSC2 but upstream of TOR itself. The GTP loading of Rheb was unchanged upon activation of MEKK3-ER, suggesting that p38 acts downstream of, or in parallel to, Rheb (Fig. 5H). The interaction between Rheb and mTOR or between mTOR and Raptor was similarly unaffected (Fig. 5I). Among all the known components of TORC1, there exists a single conserved p38 phosphorylation site, at S863 on Raptor. Commercially available phosphospecific antibodies raised against this site show that phosphorylation of this site is not modified by anisomycin treatment (Fig. 5J). In addition, p38 α , p38 γ , and MK2 are not able to significantly phosphorylate purified TORC1 in kinase assays, arguing against a role for direct phosphorylation of TORC1 components by any of these kinases (Fig. 5K and data not shown).

p38 pathway mutants in *Drosophila* are sensitive to stress and low-nutrient conditions. Having identified the p38 pathway as a regulator of growth and cell size in cultured cells, we next sought to examine the contribution of p38 signaling to cell growth *in vivo* by generating *Drosophila* strains containing p38 pathway mutations. Mutants with mutations of *p38a* are sensitive to a range of stresses (16). These flies show sensitivity to high temperature, dry starvation, and hydrogen peroxide but are not sensitive to high salt or bacterial infection (16). Interestingly, a null mutation in *mekk1*, one of at least four upstream activators of p38, results in sensitivity to both high temperature and high salt, suggesting that osmolarity acts through a kinase other than p38a (32). To further characterize this pathway, we generated null mutations for both *p38b* and its upstream kinase, *lic*, by imprecise excision of P elements.

The GenExel P element GE1091 is located within the 5' untranslated region (UTR) of the *lic* mRNA. An imprecise excision of this P element produced an allele, *lic*^{d13}, with 1,411 nucleotides removed, including those encoding the initiating

methionine and the first 351 nucleotides of the *lic* coding sequence (Fig. 6A). No *lic* transcripts are detected in *lic*^{d13}/*Y* larvae (*lic* null) (Fig. 6C). In agreement with previous work using a deletion for *lic* and the neighboring gene, *hep*, the *lic*^{d13} allele is lethal (65). *lic*^{d13}/*Y* (*lic* null) flies die 96 to 120 h after egg lay, around larval stage L3. The expression of *lic* cDNA from a transgenic construct rescues this lethality, suggesting that the adjacent gene, *hep*, is intact, since *hep* is an essential gene (26).

p38b is comprised of two exons, the first of which contains exclusively 5'UTR. An imprecise excision of P-element GE3128 located between these two exons generated a null allele, *p38b*^{d27} (Fig. 6B). This 862-bp deletion removes the first exon and half of the second, including the nucleotides coding for the initiating methionine and the first 539 nucleotides of the coding sequence. No *p38b* transcript is detected in homozygous *p38b*^{d27} (*p38b* null) flies (Fig. 6D).

Unlike the viable *p38a* null allele, the *p38b* null allele is semilethal when homozygous (Fig. 6G). Approximately 20% of *p38b*^{d27} homozygous larvae die before pupariation, with an additional 20% of homozygous pupae failing to emerge. This suggests a nonredundant role for p38b during development, during both larval and pupal stages. We used an antibody raised against phosphorylated human p38 to investigate the phosphorylation status of p38 in developing larvae. This antibody reacts with human p38 α , - β , and - γ and detects phosphorylated p38 in *Drosophila* S2 cells upon stimulation with known p38-activating stresses (76). This antibody is predicted to detect both phosphorylated p38a and p38b. Interestingly, in the *lic* null larvae and the *p38b* null larvae collected 72 h after egg lay, little phosphorylated p38 is detected, whereas larvae homozygous for the *p38a* null allele have levels of phospho-p38 similar to the levels in *w*¹¹¹⁸ and *gfp* control larvae (Fig. 6E). *p38a* and *p38b* are not completely redundant; while *p38a* mutants are viable and *p38b* mutants are semilethal, *p38a,b* double mutants are lethal and die around the same time as *lic* null larvae (Fig. 6F).

The above-described data suggest that *p38a* and *p38b* have both overlapping and distinct functions. Studies of the stress sensitivities of p38 pathway mutants are consistent with this. As mentioned above, *p38a* mutants and mutants with mutations of the upstream *Mekk1* are sensitive to partially overlapping sets of stresses (16, 32). This suggests not only that activation of p38 may occur through different upstream members of this pathway but also that p38a and p38b are nonredundant in these responses. To differentiate between different types of stress, flies null for *p38a* or *p38b* or heterozygous for *lic* were subjected to environmental stresses. Similar to the *Mekk1* mutant, *p38b* mutant embryos die under high-salt conditions (Fig. 7A). Consistent with previous results, the *p38a* mutant embryos are not sensitive to high salt. Both *p38a* and *p38b* mutants are sensitive to H₂O₂, dry starvation, and high temperatures (Fig.

anisomycin (an), or amino acids (aa) was performed for 20 min before cells were lysed. Immunoprecipitation was performed with anti-HA antibodies, and immunoprecipitates were analyzed by Western blotting. (K) 293 cells stably transfected with either empty vector (V) or TAP-tagged Raptor (Rap) were lysed, and tandem affinity tag purification was performed. These purified complexes were then incubated with purified, recombinant p38 α or p38 γ along with [³³P]ATP. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by phosphorimager. aa, amino acids; an, anisomycin; V, empty vector; dn, dominant negative Rag; i.p. or I.P., immunoprecipitates.

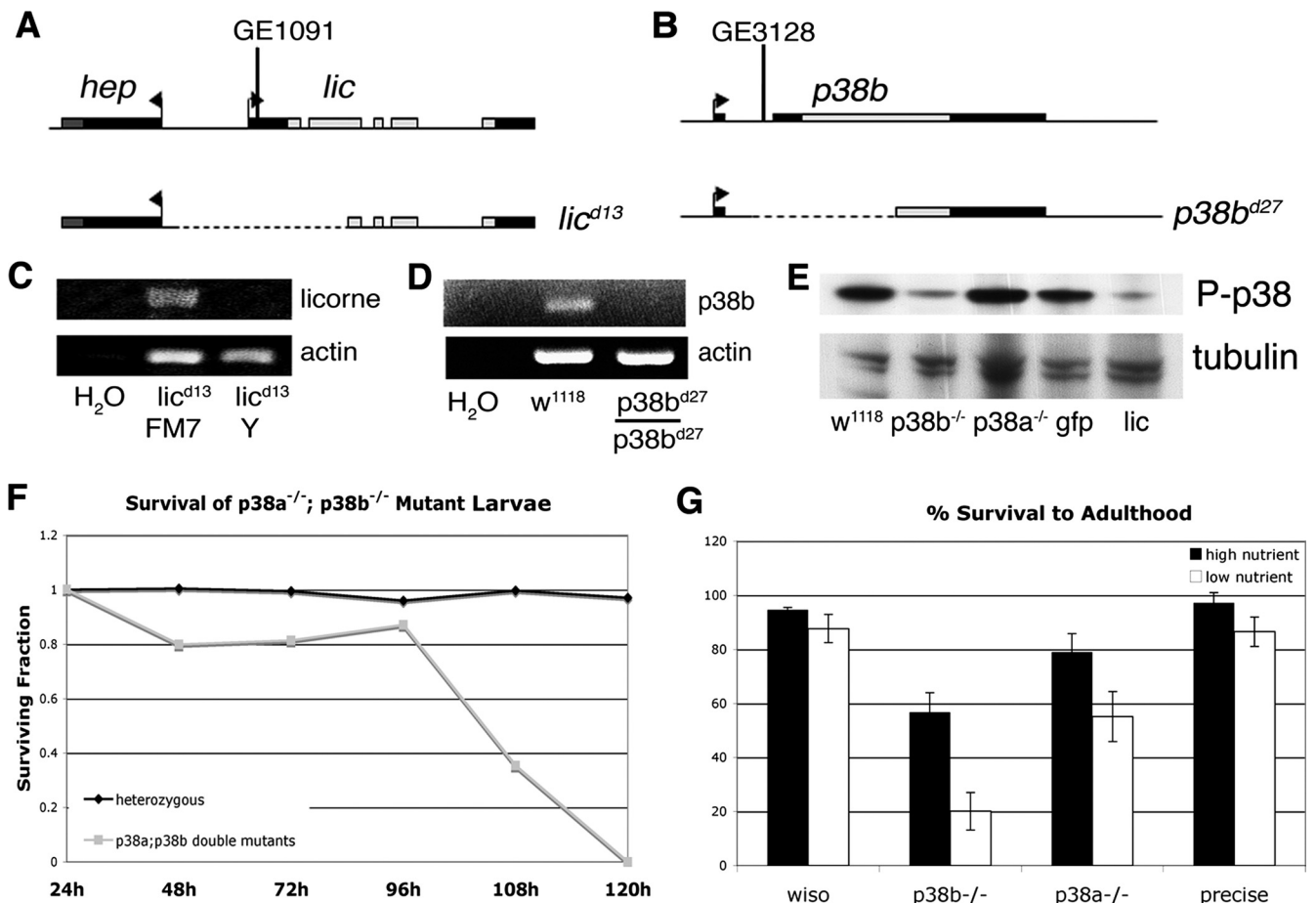


FIG. 6. Genetic disruption of *licorne* and *p38b*. (A, B) Schematic diagrams showing the genomic regions surrounding *licorne* (A) and *p38b* (B). Imprecise excisions from these loci generated null alleles, *lic^{d13}* and *p38b^{d27}*, diagrammed below. (C, D) RT-PCR results from larvae of the indicated genotype. (E) Western blot analysis of phospho-p38 levels in larvae of the indicated genotypes harvested 72 h after egg lay. (F) Survival of larvae null for both *p38a* and *p38b*. Larvae were counted every 24 h. (G) Fraction of larvae of the indicated genotypes that survived to adulthood. L1 larvae were collected 24 h after egg lay and transferred in groups of 50 to vials containing either high-nutrient or low-nutrient food. The total number of adults eclosing from each vial in the subsequent 14 days were counted. Error bars indicate standard deviations. *wiso*, *w^{iso}*; *precise*, *precise* excision.

7B to G). Mutants heterozygous for *lic* were not sensitive to any of the stresses tested, suggesting that *lic* is not haploinsufficient under these conditions (Fig. 7).

As discussed above, TORC1 responds to amino acids, and the TOR pathway mutants are sensitive to nutritional status. Importantly, *p38* pathway mutants are also nutritionally sensitive. Flies null for *p38b* have a partially lethal phenotype, as approximately half of the larvae null for *p38b* die during development (Fig. 6G). Interestingly, this lethality can be accentuated by raising the larvae on a low-nutrient medium (Fig. 6G). Under these conditions, only 20% of *p38b* larvae survive to adulthood. Furthermore, larvae null for *p38a*, which have a very mild lethality when nutrients are abundant, display a significant developmental lethality when raised on low-nutrient food. Thus, larvae with mutations of either *p38a* or *p38b* are hypersensitive to conditions in which nutrients are limited.

Disruption of p38 signaling in *Drosophila* reduces cell size. *lic* mutant larvae are strikingly reduced in size for their developmental stage (Fig. 8A). To investigate whether this body size phenotype reflects a reduction in cell size, we generated mo-

saic tissue using the FLP-FLP recombination target (FRT) system. We generated mosaic wing discs containing both *lic* null clones in *lic* heterozygous tissue and *p38b* null clones in *p38b* heterozygous tissue. To reduce any effects due to compensation from *p38a*, the *p38b* mosaic wing discs were generated in flies genetically null for *p38a*. These clones were analyzed by flow cytometry (Fig. 8B). Consistent with the phenotypes seen in *lic* null larvae, both *lic* null cells and *p38b* null cells were approximately 15% smaller than heterozygous cells from the same tissue. No differences in cell cycle profiles were observed (data not shown). Thus, similar to reduction of dTOR signaling, ablation of the p38 signaling pathway has a cell-autonomous effect on wing disc cell size.

The lethality of *lic* null larvae precludes the analysis of *lic* null adult flies, so instead we examined the size of *p38b* null adults. Significantly, adult flies null for *p38b* are also small (Fig. 8D and E). Each cell in the wing blade secretes a single hair, or trichome, and the density of these hairs therefore reflects cell size in the wing. The *p38b* size phenotype appears to be primarily due to a decrease in cell size, as bristle density on the

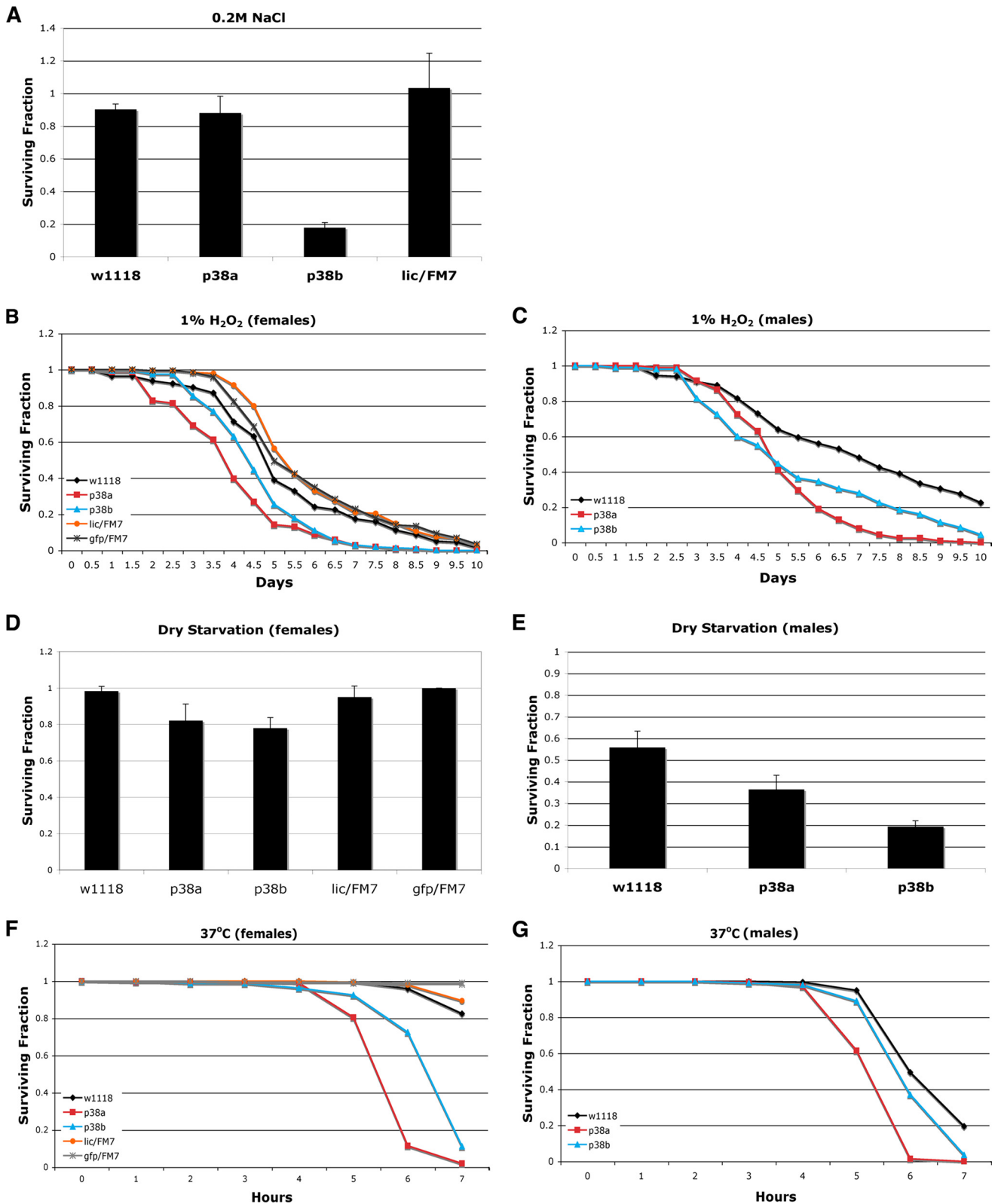


FIG. 7. *p38b* and *lic* mutants are sensitive to certain stresses. (A) *p38b^{d27}* larvae are sensitive to high salt. Larvae of the indicated genotypes were collected 24 h after egg lay and placed on normal food supplemented with 0.2 M NaCl. Surviving adults were counted 12 days later. Error bars indicate standard deviations; $n = 225$ per genotype. (B, C) *p38b^{d27}* adult flies are sensitive to H₂O₂. Two- to five-day-old females (B) and males (C) of the indicated genotypes were placed in vials containing normal food supplemented with 1% H₂O₂. Surviving flies were counted every 12 h. $n = 200$ per genotype. (D, E) *p38b^{d27}* and *lic^{d13}/FM7* adult flies are sensitive to dry starvation. Two- to five-day-old females (D) and males (E) of the indicated genotypes were placed in empty vials. Surviving flies were counted 24 h and 36 h later. Error bars indicate standard deviations; $n = 200$ to 250 per genotype. (F, G) *p38b^{d27}* adult flies are sensitive to high temperatures. Two- to five-day-old females (F) and males (G) of the indicated genotypes were placed in vials containing wet paper towel and placed at 37°C. Surviving flies were counted every 30 min. $n = 200$ per genotype.

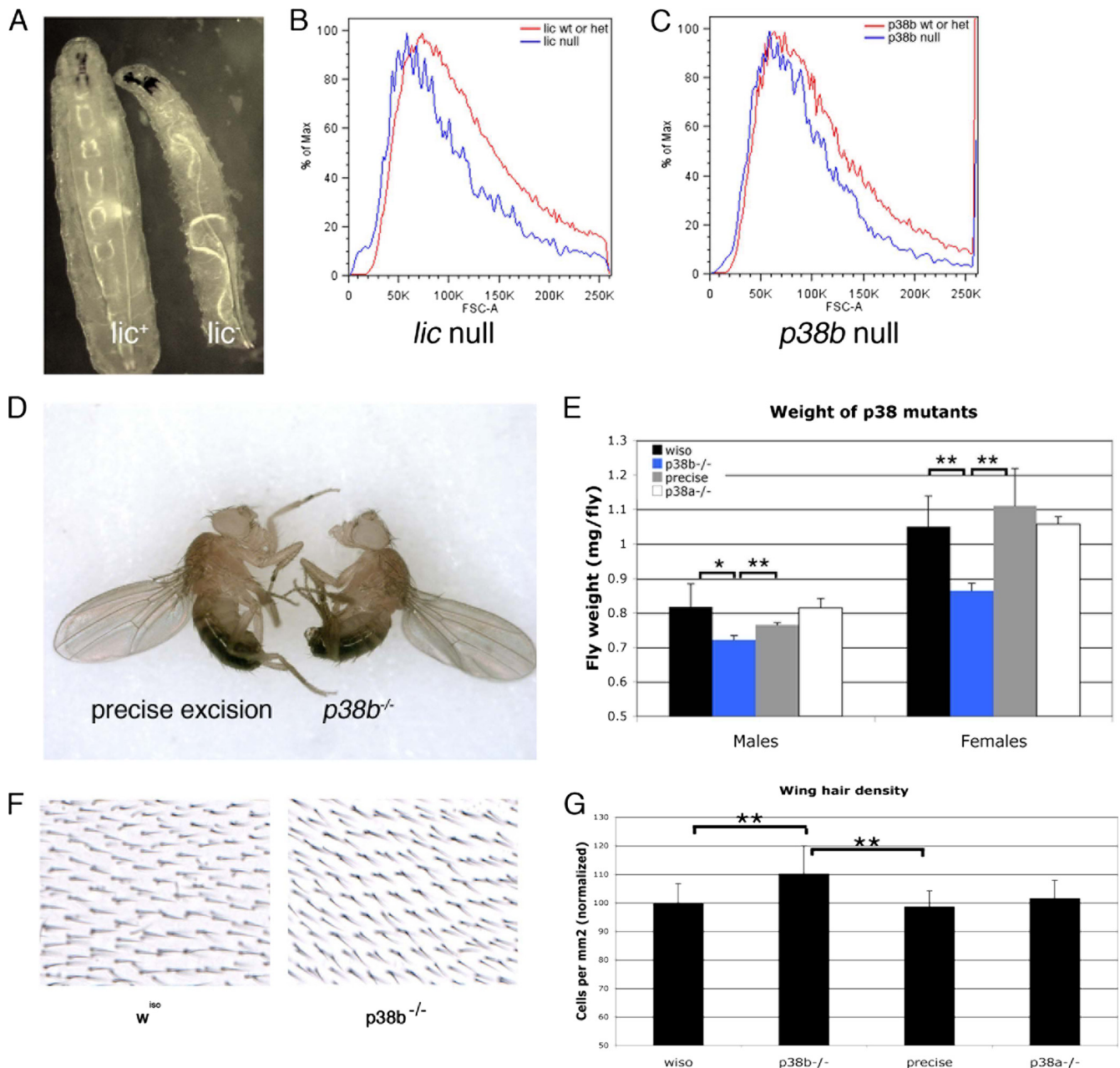


FIG. 8. Genetic disruption of *licome* or *p38b* decreases cell and organism size. (A) Phenotype of *lic*^{d13} larvae from density-controlled vials 72 h after egg lay. (B, C) Results of flow cytometry analysis of wings discs containing *lic* null cells (B) and of wing discs containing cells null for both *p38a* and *p38b* (C). wt, wild type; het, heterozygous. (D, E) Phenotype of *p38b*^{d24}/*p38b*^{d24} adults. Flies with the indicated genotypes were raised in low-nutrient food at a density of 50 larvae per vial. Adult flies were weighed in groups of 20. Error bars represent the standard deviations; $n > 150$ per genotype. *, P value of < 0.005 by Student's t test; **, P value of < 0.001 by Student's t test. (F, G) Hair densities on wings from adult flies raised on low-nutrient food. (F) High magnification of a region posterior to the L5 vein of wings with the indicated genotypes. (G) Quantification of the hair densities shown in panel F. The number of hairs in a defined area posterior to L5 was counted. Error bars represent the standard deviations; $n = 20$. **, P value of < 0.001 by Student's t test; wiso, w^{iso}. Full genotypes are as follows. Panels A to C: *lic*^{d13}/*FM7,Kr-GFP* and *lic*^{d13}/*Y* (A); *FRT19A,lic*^{d13}/*FRT19A,Ub-GFP; hsp70-FLP* (B); *hsp70-FLP; FRT40A,p38b*^{d27}/*FRT40A,GFP*; and *p38a*¹/*p38a*¹ (C). Panels D to G: precise excision, *p38b*^{d27}/*p38b*^{d27}; *p38b*, *p38b*^{d24}/*p38b*^{d24}; *p38a*, *p38a*¹/*p38a*¹.

wings of *p38b* null flies is increased by approximately 10% (Fig. 8F and G). Together, these data are consistent with our findings in the RNAi screen and demonstrate that genetic disruption of either *p38b* or *lic* results in cell-autonomous cell size decreases *in vivo*. Importantly, this phenotype is similar to

phenotypes previously observed in TOR pathway mutants. However, we have not been able to assess the activation state of downstream targets of TOR in either *p38b* or *lic* mutant embryos, so that the effect of these mutations on cell size seen here could be due in part to TOR-independent mechanisms.

DISCUSSION

The *Drosophila* S2 cell culture system is particularly amenable to manipulation both by RNAi and by insulin and was therefore chosen for an RNAi screen targeting the TORC1 pathway. Indeed, all three known kinases directly involved in TORC1 signaling downstream of TSC2 (TOR, PDK1, and S6K) were found to be positive regulators of cell size in our screen. There are few described kinases that act as direct negative regulators of TORC1. In addition to its role in phosphorylating TSC2, AMPK has recently been described as phosphorylating and inhibiting Raptor directly (28); AMPK is one of the few non-cell cycle genes identified in our screen as a negative regulator of cell size (see Table S1 in the supplemental material).

TORC1 regulation by p38. While p38 is necessary for TORC1 activation and p38 activation itself can induce TORC1 activity and the associated cell size changes, the mechanism through which this occurs is not fully understood. Since p38 modulation changes the phosphorylation status of both 4EBP1 and S6K, p38 is likely to act upstream of TOR (Fig. 2, 3, 4, and 5). As the screen itself relied on the use of TSC2 RNAi, the activation of TORC1 by p38 should occur downstream of or in parallel to TSC2. p38 affects cell size through two distinct mechanisms, one of which is S6K dependent and the other of which is MK2 dependent (Fig. 1 and 2). Precisely how MK2 affects cell size remains unexplored; RNAi targeting MK2 does not affect S6K phosphorylation (Fig. 2). MK2 has been described as phosphorylating TSC2, creating a 14-3-3 binding site (38). This is unlikely to be the mechanism through which the p38 pathway was identified in our screen, since TSC2 itself was reduced to undetectable levels in our S2 cells by using RNAi.

The p38 cascade and amino acid sensing. It is interesting to note that RNAi against MKK3 and MKK6 is able to prevent the phosphorylation of S6 and 4EBP1 in response to amino acids and growth factors (Fig. 5). This is reminiscent of the relationship between growth factors and amino acids; insulin is able to activate TORC1 only when amino acids are present (58). The simplest explanation for this is that amino acids themselves activate p38. In support of this theory, a recent report has shown activation of p38 by amino acids (14). MAP4K3, a kinase activated by amino acids, has homology to other MAP kinases which activate p38, Jun N-terminal protein kinase (JNK), or MEK (22). MAP4K3, however, appears to activate the JNK stress signaling pathway specifically, with little activity toward the p38 cascade (18). We were unable to see a robust, reproducible phosphorylation of p38 in response to either amino acids or insulin (Fig. 3 and 5 and data not shown). Thus, MAP4K3 is likely to activate targets other than p38 in order to induce TORC1 activity in response to amino acids. While p38 phosphorylation is undetectable in unstimulated mammalian cells, some basal level of activity must be present and required for TORC1 activity. Interestingly, in *Drosophila* systems, basal p38 phosphorylation is detectable (Fig. 3A and B), and in these cells, RNAi against *Licorne* affects cell size even when the TORC1 pathway is not activated.

To date, much of the evidence linking stresses to TORC1 suggests that stresses inactivate TORC1. For example, hypoxic stress inactivates TORC1 through the phosphorylation of TSC2 by Redd1 (8, 17, 53, 63), energetic stress inactivates

TORC1 through the activation of AMPK (15, 28, 31, 63), and the treatment of cells with antibiotics inactivates TORC1 through undefined mechanisms (33). These stresses are all independent of p38. In contrast, UV radiation, which activates stress pathways such as p38 and JNK through the induction of DNA damage, activates TORC1 in a number of cell types (7, 48, 75).

Thus, it appears that, when faced with cellular damage or stress, cells can respond by shutting down cell growth, allowing repair to take place until the cell commits to further growth and division. Alternatively, cells can promote growth and translation, presumably in order to promote the synthesis of stress response proteins and the turnover of damaged molecules. Our data are consistent with the hypothesis that activation of TORC1 in response to stress is dependent on the type, intensity, and duration of the incident stress and on the specific pathways activated by each (Fig. 3). The relationship between p38 phosphorylation and TORC1 activation is not linear. For example, only low doses of H₂O₂ elicit TORC1 activation (Fig. 3D), suggesting that, beyond a certain damage threshold, increased translation is not a desirable response to oxidative damage. It is therefore possible that under low levels of stress, the appropriate biological response in many cell types involves attempts to combat the incident stress, while high levels of stress or prolonged exposure to stress would induce a response focused on energy conservation.

There are at least two examples of situations in which stresses, via p38, would be predicted to increase growth and translation. First, p38 is a critical component of the immune response to infection, both in antigen-presenting cells and in T cells (1). This response involves p38-dependent increases in both transcription and translation of key cytokines. Increased translation of existing mRNAs occurs at least partially through the stabilization of mRNAs containing 3' AU-rich elements, although other p38-dependent effects on translational machinery are also observed (2). It is not currently known how increased mRNA stability is achieved or whether there is increased translation of these mRNAs in addition to the effects seen on stability. It is possible that p38 stimulates cytokine production partially through the activation of TORC1, which may contribute to increased translation of cytokine mRNAs. It is noteworthy that the main clinical use of rapamycin is as an immunosuppressant. Rapamycin treatment alters a number of immunological responses, including causing decreased cytokine production by professional antigen-presenting cells (12). Some of the immunosuppressant effects of rapamycin may be due to the inhibition of TORC1 in response to p38 activation.

Numerous growth factors, including known TORC1 activators, such as insulin and EGF, induce the production of reactive oxygen species (ROS). ROS activate p38 (19). As a by-product of mitochondrial respiration, ROS are also produced by metabolically active cells. Interestingly, chemical disruption of mitochondrial energetics (and therefore reduced ROS production) results in the dephosphorylation of TORC1 targets; this can be reversed by simultaneously treating cells with oxidizing compounds (61). Consistently, in hepatic stellate cells, ROS are required for TORC1 activation in response to amino acid treatment (46). Mitochondrial respiration results in the production of ROS; ROS may therefore act as a sensor for mitochondrial capacity. In this context, activation of TORC1

by ROS may be a mechanism through which a cell is able to couple its ability to generate energy with translation rates via p38, only permitting protein synthesis when energy levels are sufficient.

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