Identification of potential drug targets for tuberous sclerosis complex by synthetic screens combining CRISPR-based knockouts with RNAi

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The tuberous sclerosis complex (TSC) family of tumor suppressors, TSC1 and TSC2, function together in an evolutionarily conserved protein complex that is a point of convergence for major cell signaling pathways that regulate mTOR complex 1 (mTORC1). Mutation or aberrant inhibition of the TSC complex is common in various human tumor syndromes and cancers. The discovery of novel therapeutic strategies to selectively target cells with functional loss of this complex is therefore of clinical relevance to patients with nonmalignant TSC and those with sporadic cancers. We developed a CRISPR-based method to generate homogeneous mutant Drosophila cell lines. By combining TSC1 or TSC2 mutant cell lines with RNAi screens against all kinases and phosphatases, we identified synthetic interactions with TSC1 and TSC2. Individual knockdown of three candidate genes (mRNA-cap, Pitsirp, and CycT; orthologs of RINGT, CDK11, and CCNT1 in humans) reduced the population growth rate of Drosophila cells lacking either TSC1 or TSC2 but not that of wild-type cells. Moreover, individual knockdown of these three genes had similar growth-inhibiting effects in mammalian TSC2-deficient cell lines, including human tumor-derived cells, illustrating the power of this cross-species screening strategy to identify potential drug targets.

INTRODUCTION

The tuberous sclerosis complex (TSC) family of tumor suppressors, TSC1 and TSC2, function together in an evolutionarily conserved protein complex that is a point of convergence for major cell signaling pathways that regulate mTOR complex 1 (mTORC1). Mutation or aberrant inhibition of the TSC complex is common in various human tumor syndromes and cancers. The discovery of novel therapeutic strategies to selectively target cells with functional loss of this complex is therefore of clinical relevance to patients with nonmalignant TSC and those with sporadic cancers. We developed a CRISPR-based method to generate homogeneous mutant Drosophila cell lines. By combining TSC1 or TSC2 mutant cell lines with RNAi screens against all kinases and phosphatases, we identified synthetic interactions with TSC1 and TSC2. Individual knockdown of three candidate genes (mRNA-cap, Pitsirp, and CycT; orthologs of RINGT, CDK11, and CCNT1 in humans) reduced the population growth rate of Drosophila cells lacking either TSC1 or TSC2 but not that of wild-type cells. Moreover, individual knockdown of these three genes had similar growth-inhibiting effects in mammalian TSC2-deficient cell lines, including human tumor-derived cells, illustrating the power of this cross-species screening strategy to identify potential drug targets.

RESULTS

Optimization of the CRISPR system for Drosophila cell culture

CRISPR functions with high efficiency in many organisms, including Drosophila (11–19), making it an ideal system for generating mutant cell lines for combinatorial screening. However, our ability to predict off-targets and short guide RNA (sgRNA) efficacy before testing is currently limited, and it
is unclear whether design rules from mammalian systems are transferable to Drosophila cells (20–23). We therefore decided to assess the specificity of CRISPR in Drosophila cell culture. We first generated a vector encoding both Cas9 and sgRNA (Supplementary file 1) and then used this to express 75 variants of an sgRNA in S2R+ cells with different mismatches to a single target sequence present in a luciferase-based reporter (Supplementary file 2) or in the genome. The extent and position of mismatch required to prevent mutation was assessed by measuring changes in luciferase expression from the reporter construct (Fig. 1A) or using high-resolution melt assays (HRMAs) on endogenous sequences (fig. S1). Both approaches produced similar results that are consistent with previous observations (13, 24). For example, in previous reports from mammalian systems (21), mismatches at the 5′ end of the sgRNA sequences were better tolerated than those at the 3′ end. However, in some cases, a single mismatch was sufficient to prevent detectable mutation. In addition, we found that three mismatches were sufficient to prevent detectable mutations except when all mismatches were at the 5′ end of the sgRNA, consistent with a previous report investigating the specificity of CRISPR in vivo in Drosophila (25). We therefore used 3 base pairs (bp) of mismatch as a cutoff to annotate predicted off-targets for all possible sgRNAs in the Drosophila genome and included these data in an updated version of our previously reported sgRNA design tool (www.flyrnai.org/crispr2) (fig. S2) (16). Note that to be included in this tool, sgRNAs must have a unique 3′ seed sequence. As such, no annotated sgRNA can have off-targets with mismatches clustered at the 5′ end. Using these updated off-target predictions, we estimate that 97% of genes in the Drosophila genome can be targeted with specific sgRNAs, making this an ideal system for the generation of knockout cell lines.

Because the rate of mutations varies widely between different sgRNAs (26–28), we tested whether efficiency could be predicted on the basis of the sgRNA sequence. We generated 75 additional sgRNAs each targeting luciferase-based reporter constructs with no mismatches and tested mutation efficiency for each (table S1). Using this panel of sgRNAs and associated efficiencies, we first considered whether GC content correlated with mutation rate as has been suggested in several previous reports (25, 27, 29, 30). In contrast with the results of Ren et al. (25) suggesting that greater than 50% GC content in the six PAM (protospacer-adjacent motif)–proximal nucleotides is associated with high efficiency, we found no such correlation for any part of the sgRNA sequence (fig. S3, A to C). However, our observations are consistent with a mammalian study suggesting that both high and low GC content at the 3′ end are associated with low efficiency [fig. S3B and (27)]. Next, we tested whether a more general sequence-based approach could improve efficiency prediction. We analyzed the nucleotide content of all 75 sgRNAs considering each position separately and generated a probability matrix linking nucleotide content with mutation rate (Fig. 1B), which was used to predict efficiency scores based on sgRNA sequence. To test the performance of this approach, we generated scores for sgRNAs used in three previous Drosophila publications and found a strong correlation with reported efficiencies for two of them (Fig. 1C). Note that sgRNAs unlikely to produce a mutant phenotype (targeting close to the 3′ end of genes) or with apparent viability effects (few emerging adults) were not included in this analysis. However, very little correlation was detected for a third data set (fig. S3D). In addition, the criteria that we identified for high sgRNA efficiency differ from those of two studies performed in mammalian systems (26, 27), and these two studies also differ from the GC requirements identified previously in Drosophila (25), suggesting that in some cases, efficiency criteria may depend on factors other than simply sgRNA sequence. Finally, we generated predicted scores for all sgRNA target sites in the Drosophila genome on the basis of our findings and annotated these in our online design tool (www.flyrnai.org/crispr2) (fig. S2). With this updated tool, sgRNAs can be quickly designed for various applications.

**Generation of stable mutant cell lines**

The CRISPR system works efficiently in Drosophila cell culture (11, 12). However, it has not yet been possible to generate cell lines in which all cells are null mutants for the target gene, because previous studies have shown that mutant populations quickly revert back to wild type. To solve this issue, we first generated optimized sgRNAs to maximize their efficiencies while avoiding off-target effects (Fig. 1 and fig. S2). In addition, we implemented a method to predict the frameshift and in-frame mutation rates for each sgRNA target site (31) and annotated each of these mutation rates in our
online design tool (www.flyrnai.org/crispr2). Second, to ensure that mutant cell lines do not revert to wild type, a method is required to grow cultures from individual cells, a historically difficult problem with *Drosophila* cells. Various methods for this problem have been proposed (32–34), but none have been widely used because of either difficulty in identifying single cell–derived cultures or very low efficiencies. To substitute for paracrine factors that promote the survival of individual *Drosophila* cells cultured in populations, we tested whether the use of culture media preconditioned with wild-type S2R+ cells would allow the efficient growth of individual S2R+ cells isolated by flow cytometry. When seeded into regular media, 0 of 190 individual cells formed colonies, but when seeded into conditioned media, 30 of 190 (16%) cells formed colonies that could be expanded into clonal cultures (Fig. 2A). Varying the fetal bovine serum (FBS) concentration had no additional effect.

One difficulty associated with the isolation of mutant cells from *Drosophila* S2R+ cells is that they are aneuploid, containing roughly four copies of any given genomic locus (35). Thus, the chances of identifying cells in which all alleles carry frameshift mutations are considerably lower than for diploid cells. To assess the ability of CRISPR to produce homozygous mutations in these cells, we targeted the *yellow* gene and tested 30 individual cells for the presence of mutations using high-resolution melt (HRM) assays. Twenty-one (70%) carried mutations at the target locus (Fig. 2B).

The eight samples with the strongest signal in the HRM assays were analyzed by sequencing. No wild-type sequences were detected for any of these samples, and seven of eight contained a single mutation in all derived sequences (fig. S4). The identification of homozygous mutations in seven of eight cells tested is consistent with previous reports of high rates of gene conversion after genome editing (36–38); however, it is also possible that homozygous mutations are generated as a result of chromosome loss. Therefore, the HRM assay is an effective method to identify fully mutant clones.

Next, to test the efficacy of our sgRNA design tool and the combined CRISPR and single-cell cloning approach (fig. S5A), we targeted a gene for which loss of protein function could easily be assayed: *STAT92E*, which encodes a STAT (signal transducer and activator of transcription) transcription factor that is activated by JAK (Janus kinase) (fig. S5B). Fifteen clones were analyzed, of which 13 carried mutations on all alleles. Further testing showed that the expected phenotype was produced from these knockouts, with the *STAT92E* line unable to respond to JAK/STAT pathway stimulation induced by *upd* ligand overexpression (Fig. 2C). In addition, the effect of the *STAT92E* mutation was considerably stronger than that produced by targeting *STAT92E* with double-stranded RNA (dsRNA), which reduced the response to JAK/STAT pathway stimulation but did not prevent it. These results demonstrate that our approach provides an efficient CRISPR-based method for the production of stable, homogenous mutant *Drosophila* cell lines.

**Synthetic screens using TSC1 and TSC2 mutant lines**

We generated cell lines carrying frameshift mutations in the *TSC1* and *TSC2* genes using the approach described above (fig. S6). To characterize the lines, we tested whether they showed phenotypes similar to those previously reported in vivo or in mammalian cell lines (39–43) because antibodies against *Drosophila* TSC1 or TSC2 are not available. Three phenotypes were considered: cell size, responsiveness to growth factor deprivation, and phosphorylation of the downstream TOR target S6 kinase (*S6K*). *TSC1* and *TSC2* cell lines had all three phenotypes: an increased cell diameter (Fig. 3, A to D), an inability to modify population growth in the absence of growth factors (Fig. 3E), and increased phosphorylation of *S6K* (when normalized to *β*-tubulin; Fig. 3, F and G). To further characterize the mutant cell lines, we performed phosphoproteomic analysis. One hundred twenty-eight phosphosites showed a more than 1.5-fold increase or decrease in both mutant lines compared to wild-type cells (table S2). Gene ontology (GO) analysis demonstrated that 20 of the top 30 most significantly enriched categories were consistent with known functions of the TSC network (Fig. 3H and table S3), including insulin signaling, response to nutrients, and the growth of cells and tissues. Together, these results suggest that the cell lines accurately represent TSC mutant models.

Next, to take advantage of the homogeneous *TSC1* and *TSC2* mutant cell lines, we performed a combinatorial RNAi screen of all *Drosophila* kinases (376) and phosphatases (159). We measured population viability using a total adenosine triphosphate (ATP) readout to capture changes in cell growth, proliferation, and cell death (referred to as “population growth” from here on). Any samples with significant effects on the population growth of wild-type cells were discarded to identify TSC-specific hits. Twenty of the remaining knockdowns had significant effects on *TSC1* mutant cells, and 49 hits significantly affected *TSC2* mutant cells (Fig. 4A and table S4). Because *TSC1* and *TSC2* act as part of a protein complex and

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**Fig. 2. Generation of mutant cell lines.** (A) Survival rates of single S2R+ cells seeded into different medium formulations. “Clones” represents the number of seeded samples that produced viable populations of cells 3 weeks after seeding. Schneider’s medium was supplemented with FBS at the indicated concentrations and was preconditioned using S2R+ cells where indicated. (B) HRM results for single S2R+ cells from a population 4 days after treatment with CRISPR targeting the *yellow* gene. The graph shows the difference in fluorescence between each sample and a mean control curve against temperature (scale from 76° to 84°C). (C) Graph showing relative firefly luciferase activity normalized to *Renilla* luciferase activity for either wild-type (black bars) or *STAT92E* (gray bars) mutant cells in the presence or absence of JAK/STAT pathway activation (*upd* ligand overexpression) and with or without activation in the presence of two different dsRNAs targeting *STAT92E* (RNAi-1 and RNAi-2). Bars show the mean from five biological replicates; error bars represent SEM. All differences between wild-type and *STAT92E* cells were significant (*P* < 0.05).
mutations in either gene give rise to the TSC disease, we decided to consider for further studies genes that were identified in both the TSC1 and TSC2 screens. This approach filtered out the noise associated with either individual screen and identified genes with the most robust synthetic interactions with the TSC complex. The knockdown of three genes (mRNA-cap, Pitsre, and CycT) showed robust and specific effects on TSC1- and TSC2-deficient cells (Fig. 4A, purple crosses).

The first candidate, mRNA-cap, is the 5' triphosphatase and guanylyltransferase that catalyzes the first two steps required for the formation of a 5'-7-methylguanylate mRNA cap, which is necessary for the initiation of cap-dependent translation (44). Because activation of mammalian TOR (mTOR) promotes cap-dependent translation initiation through multiple downstream targets (45, 46), our findings suggest that TSC mutant cells depended on mRNA capping, an event that precedes the steps in translation regulated by mTOR. Our phosphoproteomic analysis identified phosphosites on distinct components of the translation initiation machinery, such as Thor, eIF4G, eIF3-S10, and eIF2B, being either increased or decreased in either TSC1, TSC2, or both mutant cell lines compared to control. In addition, phosphorylation changes were detected in both cell lines for two other proteins that directly interact with core components of the translation initiation complex (Ens and Map205, table S2) (47, 48).

Given the link between TSC signaling and translation initiation, we tested whether another translation initiation component showed a similar synthetic relationship with the TSC mutant cell lines. We knocked down eIF3-S4 in wild-type and TSC1 or TSC2 mutant cells using the same assays as for the kinase and phosphatase screen. Both TSC1 and TSC2 mutant cells had a synthetic decrease in population growth (Fig. 4A, purple circle), suggesting that the control of cap-dependent translation initiation may be a promising therapeutic target for TSC-dependent and/or mTOR complex I (mTORC1) hyperactive disease.

The second candidate, CycT, is a kinase implicated in the regulation of transcriptional elongation (49, 50). mRNA-cap is recruited to the RNA polymerase II (Pol II) C-terminal domain phosphorylated at Ser2 to form the 5'-mRNA cap, and CycT promotes RNA Pol II phosphorylation at this site. Thus, the function of CycT may be related to that of mRNA-cap. Finally, the third candidate, Pitsre, is a cyclin-dependent kinase that has been implicated in the regulation of autophagy (51). Disruption of the TSC complex leads to reduced autophagy, which has been exploited as a potential therapeutic strategy by combining autophagy inhibitors such as chloroquine with mTOR inhibitors (52).

To determine whether the identified interactions extended to CG6182 (TBC1D7 in mammals), a third component of the TSC complex (53), we

**Fig. 3. Characterization of TSC mutant cell lines.** (A to C) Images of representative fields from wild-type (A), TSC1 mutant (B), or TSC2 mutant (C) cell lines. All images were taken at the same magnification and using the same settings. Scale bars, 50 μm. (D) Graph showing frequency of cell sizes for the cell lines indicated, divided into “low diameter” (gray bars) or “high diameter” (black bars) using a cutoff at which most wild-type cells fall into the low-diameter category. Bars represent the mean from three biological replicates; error bars indicate SEM. (E) Graph showing the relative rates of population growth for the indicated cell lines in either complete medium (10% FBS; blue bars), under partial serum starvation conditions (1% FBS; red bars), or under complete serum starvation conditions (0% FBS; green bars). Note that these values represent a combination of growth and proliferation. Bars show the mean of 24 samples per cell line and condition; error bars represent SEM. (F) Images of Western blots stained for phosphorylated S6K (p-S6K) or α-tubulin as indicated. Samples represent biological triplicates from S2R+, TSC1, and TSC2 cells. p-S6K amounts were normalized to α-tubulin because an antibody for Drosophila total S6K was not available. (G) Quantification of p-S6K for the indicated cell lines as shown in the Western blots in (F). Bars represent mean change in p-S6K normalized to α-tubulin for three biological replicates in each case. Error bars represent SEM; asterisks indicate significant differences from control (P ≤ 0.01), determined by t-tests. (H) Graph indicating the fold enrichment of the indicated GO categories in phosphoproteomic data from TSC1 and TSC2 mutant cells compared to wild type. All samples are enriched with P values <0.05.
tested whether combinatorial knockdown of mRNA-cap, CycT, or PitSlre with CG6182 knockdown produced greater reduction in population growth than either knockdown alone. In all three cases, the combination of dsRNAs targeting the candidate gene and CG6182 produced a synthetic reduction in population growth (Fig. 4B).

Conservation of synthetic interactions in mammalian cells
Because all three candidates from the Drosophila screens have orthologs in mammals (table S5), we tested whether the synthetic interactions of mRNA-cap, PitSlre, and CycT with TSC1 and TSC2 were conserved. We used small...
interfering RNAs (siRNAs) targeting the orthologs of each of the three genes in TSC2-deficient MEFs compared to littermate-derived wild-type MEFs. Both RNGTT (mRNA-cap in *Drosophila*) and CCNT1 (Cyclin T in *Drosophila*) knockdowns caused reduced population growth in TSC2−/− cells compared to wild type, although no synthetic effect was detected with CDK11 (Pilsen in *Drosophila*) because CDK11 knockdown reduced mTORC1 activity in both wild-type and TSC2−/− MEFs (Fig. 4C and figs. S7, A to F; and S8). We noted, however, that similar synthetic interactions were not detected when any of the three candidates were knocked down in combination with TSC1, TSC2, or TBC1D7 using combinatorial siRNA treatments (fig. S9). These results suggest that although the target proteins were efficiently reduced (fig. S8), either residual protein was sufficient to restore some function or long-term effects of loss of function of the TSC complex were required. In the latter case, although TSC proteins were depleted, the effect might not be detectable because the cells might still have enough of the components normally regulated by TSC. Further, to assess the relevance of these potential drug targets to human tumor cells, we used siRNA to knock down the three hits in a TSC2-deficient human renal angiomyolipoma (AML) cell line derived from a patient with LAM (54). For isogenic comparison, the candidate genes were knocked down using siRNA in the same cell line stably reconstituted with wild-type TSC2. To assess the effectiveness of the TSC2 addback, we measured S6K phosphorylation and cell size with and without TSC2 reconstitution. As expected, TSC2 addback reduced mTORC1 activity and cell size (fig. S10). siRNAs targeting each of the three candidate genes significantly reduced the population growth of TSC2 null cells as assessed by using total ATP as a readout (Fig. 4D and fig. S7, G to I). In addition, synthetic effects were seen on cell numbers for all three genes (Fig. 4E). In contrast, two negative control genes that did not score in the *Drosophila* screen [Src42A (FRK in mammals) and for (PRKG1 in mammals)] showed no synthetic effects (fig. S7, J and K), indicating that the three gene products we have identified could be promising drug targets for TSC and LAM.

**DISCUSSION**

We have developed a synthetic screening method that combines the CRISPR genome-editing system with well-established RNAi methodologies. Previous combinatorial screens in *Drosophila* cells have been performed by treating cells with multiple RNAi reagents simultaneously (9, 10). However, whereas this approach has been used successfully, limitations of RNAi including incomplete transfection, partial knockdown, and off-target effects are compounded, leading to high false-negative and false-positive rates. A laborious and time-consuming secondary screening is therefore required to identify the robust hits from these screens.

The screening strategy we have developed offers several advantages compared to combinatorial RNAi treatment. First, by combining CRISPR-generated mutant cell lines with single RNAi reagents, we avoid much of the noise associated with dual RNAi-based screening approaches. The use of homogeneous populations of null mutant cells avoids the issues of incomplete transfection and incomplete knockdown. In addition, once generated, in-depth characterization of the mutant cell lines can be performed to establish whether off-target mutations are present. In the case of our screen, this reduction in noise as well as the comparison between two independent mutant cell lines completely avoided the need for secondary screening.

Second, in some cases, mutant cell lines may represent a considerable improvement in the quality of disease models over RNAi-mediated knockdowns. For example, diseases such as TSC are caused by loss-of-function mutations rather than by partial transient reduction in protein abundance. The establishment of a mutant cell line enables cellular adaptation to the induced mutation, likely generating a more representative cellular environment. This is illustrated by the lack of synthetic effects detected using siRNA-mediated combinatorial knockdowns in MEFs (fig. S9). One implication is that future screens performed in such adapted backgrounds may lead to hits with more reproducible effects in a therapeutic setting.

Third, previous screening strategies have required laborious secondary screening and validation of hits to identify those that are robust. Here, we have simultaneously screened two mutant backgrounds (TSC1 and TSC2). Because both of these proteins act as part of the TSC complex, similar effects are expected from the two knockouts. Therefore, by considering the overlap between these data sets, we were able to quickly identify the most robust candidates. The advantage of this approach is reflected in the fact that the identified synthetic candidates were validated in both mammalian cell types, resulting in a very rapid translation from screening in a model organism to identification of clinically relevant potential drug targets. Finally, by considering conservation between *Drosophila* and humans, we increase the likelihood of the identified effects being reproducible, an issue that has been a major limitation in previous studies (5).

Current treatments for TSC-related diseases include rapamycin and its derivatives, which function by blocking mTOR activity downstream of the TSC complex. A problem associated with this approach is that the molecular vulnerabilities caused by mutations in the TSC complex are reversed, thereby reducing the opportunities available to kill affected cells (55). The targets we have identified here offer the potential to bypass this issue. Indeed, none of the target genes affected the phosphorylation of S6K in AML cells when knocked down, and two (CCNT1 and RNGTT) had no effect on the phosphorylation of S6K in TSC2-deficient MEFs (fig. S8), suggesting that some molecular vulnerabilities caused by mTOR activation may persist after inhibition of these potential drug targets. For example, inhibition of mRNA-cap would not be expected to have direct effects on autophagy and, therefore, may maintain the energy stress associated with TSC mutations. However, further work will be required to determine whether inhibition of these factors is more efficacious than mTOR inhibition.

Finally, whereas we have used mutant cell lines to develop an improved synthetic screening method, there are many other possible applications of stable homogeneous mutant lines, for example, in modeling of diseases caused by single null mutations or epistasis experiments where residual expression of the target gene can complicate interpretation of results. We therefore expect this method to be widely applicable to many different areas of research.

In conclusion, by combining established RNAi screening methods in *Drosophila* cells with CRISPR genome-editing technology, we have developed a powerful new approach to synthetic screening. The robustness of this method is demonstrated by the conservation of the identified synthetic interactions in mouse and human systems, suggesting that it will be a generally applicable approach to investigate various biological and disease-relevant questions.

**MATERIALS AND METHODS**

**Generation of CRISPR expression vector**

A *Drosophila* codon-optimized *Cas9* with a 3xFlag tag and nuclear localization signal elements at both 5′ and 3′ was synthetized by GenScript, and the *Drosophila* U6 and act5c promoters were polymerase chain reaction (PCR)-amplified from *Drosophila* genomic DNA (table S6). These were used to replace the human codon-optimized *Cas9* and human U6 and *CG8* promoters, respectively, of the pX330 (13) plasmid to yield the pl018 plasmid (Supplementary file 1). sgRNA homology sequences were cloned into pl018 using pairs of DNA oligonucleotides, which were annealed...
and ligated into Bbs I sites according to a previously described protocol (table S6) (13).

Luciferase-based mutation reporter assays

The luciferase reporter vector was constructed by PCR amplifying the metallothionein promoter from pMK33 (56) and luciferase gene from pGLO3 (table S6) (37) and combining these with annealed oligos containing an sgRNA target site (tables S1 and S6) and a custom-made cloning vector using Golden Gate assembly. Luciferase assays were performed by transfecting S2R+ cells with the relevant pl018 plasmid, luciferase reporter, and pRL-TK (Promega) (to allow normalization of transfection efficiencies between samples) in 96-well plates using Effectene transfection reagent (Qiagen) according to the manufacturer’s recommendations. Twenty-four hours after transfection, CuSO4 was added to the cell medium at a final concentration of 140 μM, and cells were incubated for a further 16 hours. Firefly and Renilla luciferase readings were taken using the Dual-Glo Luciferase Assay System (Promega) and a SpectraMax Paradigm Multi-Mode Microplate Detection Platform (Molecular Devices) according to the manufacturers’ instructions.

Online tools

An improved version of CRISPR design tool was implemented reusing some of the modules developed previously (16). Besides allowing users to choose different off-target thresholds, this version also displays precalculated efficiency score and restriction enzyme annotation. The efficiency score was calculated on the basis of a probability matrix computed using the in vitro cell line data described in Fig. 1A. It reflects a cumulative P value for high efficiency of each nucleotide from position 1 to 20, with higher values representing higher efficiency. A user interface allowing efficiency score calculation for user-provided sequences was also developed as part of the improved tool, which dynamically calculates predicted efficiency scores for each input sequence from position 1 to 20 or over a user-defined region (fig. S2).

HRMAnalyzer was written as a series of Matlab programs running under the control of CGI front-end implemented in Perl and JavaScript. The Matlab programs are compiled as stand-alone executable programs and called from within the Perl CGI back-end script. Both tools are hosted on a shared server provided by the Research Information Technology Group (RITG) at Harvard Medical School.

Transfections

Cells were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. For generation of mutant cell lines, we used 360 ng of pl018 plasmid and 40 ng of actin-green fluorescent protein (GFP) plasmid as a marker of transfected cells. Transfections were performed in six-well plates and, unless stated otherwise, were incubated for a further 16 hours. Firefly and Renilla luciferase readings were taken using the Dual-Glo Luciferase Assay System (Promega) and a SpectraMax Paradigm Multi-Mode Microplate Detection Platform (Molecular Devices) according to the manufacturers’ instructions.

Cell size assays

S2R+, TSC1, and TSC2 mutant cell lines were analyzed using a BD Biosciences LSR Fortessa X-20 cell analyzer to measure forward scatter for each cell as a proxy for cell diameter.

Cell line growth assays

Five thousand cells from each line were seeded into 384-well plates containing 50 μl of culture medium and incubated at 25°C for 5 days. CellTiter-Glo reagent (27 μl; Promega) was added to each well before reading luminescence using a SpectraMax Paradigm Multi-Mode Microplate Detection Platform (Molecular Devices).

Quantitative phosphoproteomics

Phosphoproteomic analysis was performed as described previously (59). Briefly, S2R+, TSC1, or TSC2 mutant cells were serum-starved for 16 hours before lysis in 8 M urea. Samples were then digested with trypsin, and peptides were chemically labeled with TMT Isobaric Mass Tags (Thermo Scientific), separated into 12 fractions by strong cation exchange chromatography, purified with TiO2 microspheres, and analyzed by liquid chromatography–tandem mass spectrometry on an Orbitrap Velos Pro mass spectrometer (Thermo Scientific). Peptides were identified by Sequest and filtered to a...
1% peptide false discovery rate (FDR). Proteins were filtered to achieve a 2% final protein FDR (final peptide FDR near 0.15%). TMT reporter ion intensities for individual phosphopeptides were normalized to the summed reporter ion intensity for each TMT label. The localizations of phospho-sites were assigned using the Ascore algorithm.

**Synthetic screening**

S2R+, TSC1, and TSC2 mutant cell lines were each screened in triplicate using the “kinases and phosphatases” sublibrary provided by the DRSC (www.flymap.org). Screening was performed following standard procedures as described by the DRSC (www.flymap.org/DRSC-PRR.html). Briefly, for each 384-well plate, 5000 cells in 10 μl of FBS-free medium were seeded into each well, already containing 5 μl of dsRNA at a concentration of 50 ng/μl. Samples were incubated at room temperature for 45 min before adding 35 μl of 14% FBS medium (bringing the final FBS concentration to 10%). The plates were incubated at 25°C for 5 days before assaying ATP concentrations using CellTiter-Glo assays (Promega) and a SpectraMax Paradigm Multi-Mode Microplate Detection Platform (Molecular Devices). The CellTiter-Glo Luminescent Cell Viability Assay determines the number of viable cells in culture on the basis of quantitation of the ATP present, thus measuring changes in cell growth, proliferation, and/or cell death (population growth).

CellTiter-Glo data were analyzed by normalizing the data to the median value of each column (to correct for pipetting errors) and calculating the z-scores for each trial individually. Z-scores greater than 1.5 or less than −1.5 in at least two of three trials were considered to affect population growth significantly. Synthetic hits were identified as dsRNAs that significantly affect the population growth of TSC1 or TSC2 mutant cell lines but not S2R+.

**Validation of synthetic interactions in mammalian cells**

TSC2+/−;TP53+/− and TSC2−/−;TP53−/− MEFs (60) and TSC2-deficient AML cells with empty vector or TSC2 addback (61) were transfected with siGENOME SMARTpool siRNAs (Dharmacon) targeting TP53, using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s reverse transfection protocol. ATP concentrations were quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions. The following antibodies were purchased from Cell Signaling Technology and used for Western blot analysis: TSC2 (#3612), phospho-Thr389 S6K (#2708), GAPDH (#5174), CCNT1 (#8744), and CDK11 (#5524).

**R E F E R E N C E S  A N D  N O T E S**


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Identification of potential drug targets for tuberous sclerosis complex by synthetic screens combining CRISPR-based knockouts with RNAi
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