although these findings require further exploration in larger clinical cohorts. Additional studies of clinical response and resistance to immune checkpoint inhibitors may benefit from integrating exome and transcriptome sequencing data to inform the relative contributions of tumor immunogenicity and host immune infiltration in determining clinical benefit.

**REFERENCES AND NOTES**

18. Materials and methods are available as supplementary materials on Science Online.

**ACKNOWLEDGMENTS**

This work was supported by NIH U54 HG003067. All sequencing data are available in dbGAP, accession number phs000452.v2.p1. We thank the Dermatologic Cooperative Oncology Group (DoCOG; Germany) for clinical accrual and sample coordination. S. Bahl (Broad) for project management effort, and the Broad Genomics Platform for piloting dual extraction and RNA sequencing from formalin samples. S.M.G. is supported by a fellowship from Janssen and is a paid adviser for Merck, Bristol-Myers Squibb, Roche, and Novartis. C.L. is a paid adviser for Merck, Bristol-Myers Squibb, Roche, and Novartis. L.A.G. is a consultant for Novartis, Foundation Medicine, and Boehringer Ingelheim, a paid adviser for Warp Drive, has ownership interest in Foundation Medicine, and holds a Commercial Research Grant from Novartis. E.M.V.A. is a paid adviser for Syapse and a consultant for Roche Ventana. E.M.V.A., D.M., B.S., D.S., and L.A.G. designed the study, performed the analyses, and wrote the manuscript. S.A.S., D.M., E.M.V.A., and C.J.W., performed HLA typing and neoantigen binder analyses. E.M.V.A., S.G., and L.A.G. performed sequencing. B.S., C.B., L.Z. A.S., M.H.G.F., S.M.G., J.U., J.C.H. B.W., K.C.K., C.L., P.M., R.G., R.D., and D.S. developed the clinical samples cohort. U.H. performed pathology review on the samples.

**SUPPLEMENTARY MATERIALS**

www.sciencemag.org/content/350/6257/2517/suppl/DC1

Materials and Methods

Figs. S1 to S5
Tables S1 to S5
References (25–41)

13 July 2015; accepted 27 August 2015
Published online 10 September 2015
10.1126/science.aad0095

**ONCOGENE SIGNALING**

Identification of an oncogenic RAB protein

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In a short hairpin RNA screen for genes that affect AKT phosphorylation, we identified the RAB35 small guanosine triphosphatase (GTPase)—a protein previously implicated in endomembrane trafficking—as a regulator of the phosphatidylinositol 3’-OH kinase (PI3K) pathway. Depletion of RAB35 suppresses AKT phosphorylation in response to growth factors, whereas expression of a dominant active GTPase-deficient mutant of RAB35 constitutively activates the PI3K/AKT pathway. RAB35 functions downstream of growth factor receptors and upstream of PDK1 and mTORC2 and copurifies with PI3K in immunoprecipitation assays. Two somatic RAB35 mutations found in human tumors generate alleles that constitutively activate PI3K/AKT signaling, suppress apoptosis, and transform cells in a PI3K-dependent manner. Furthermore, oncogenic RAB35 is sufficient to drive platelet-derived growth factor receptor α to LAMP2-positive endomembranes in the absence of ligand, suggesting that there may be latent oncogenic potential in dysregulated endomembrane trafficking.

The phosphatidylinositol 3’-OH kinase (PI3K)/AKT pathway is a key regulator of cellular survival, proliferation, and growth and is commonly activated in human cancers by receptor tyrosine kinase (RTK) signaling (1, 2). To identify previously unknown regulators of PI3K/AKT signaling, we performed an RNA interference (RNAi) screen using lentiviruses that express short hairpin RNAs (shRNAs) (3) targeting genes coding for all known G proteins.
and lipid or protein kinases. Because AKT phosphorylation is a faithful indicator of PI3K activity, we used an immunofluorescent approach to quantitatively measure phosphorylation of AKT at serine 473 (S473) (4) in HeLa cells, which display robust growth factor–induced AKT phosphorylation and lack mutations in core PI3K signaling pathway genes (Fig. 1A). We screened a collection of 7450 shRNAs in an arrayed format to triplicate identify kinases or guanosine triphosphatases (GTPases) whose depletion could alter PI3K-dependent AKT phosphorylation.

To identify shRNAs that altered AKT phosphorylation (i.e., “hits”), we translated the phospho-AKT signal in each screened well to a z score (5), shRNAs targeting genes that positively regulate AKT phosphorylation (pAKT)—RICTOR, PIK3CA, PIK3CB, AKT1, and AKT3—all diminished AKT phosphorylation and scored as “pAKT-low” hits (Fig. 1B and tables S1 to S3). Conversely, shRNAs targeting genes whose protein products inhibit AKT signaling—such as RAPTOR and RHEB—elevated phospho-AKT levels and scored as “pAKT-high” hits. Thus, our screen successfully identified known regulators of PI3K/AKT signaling, as well as a number of genes not previously implicated in regulation of the pathway (table S4). We prioritized hits for genes that had no reported link to PI3K/AKT signaling (table S5) and searched oncogenic databases to identify those with somatic mutations reported in human cancers (6, 7). This left us with a list of 29 genes (table S6), 26 of which were pAKT-low hits. Next, we grouped these 26 pAKT-low hits by their annotated functions from the literature and found that the best-represented functional group was the RAB GTPases, which are well known to regulate intracellular protein trafficking (Fig. 1C). This finding suggests that dysregulated protein trafficking by mutant RAB proteins could play a role in oncogenesis.

Of the five RAB proteins that appeared in our list in table S6 (see also Fig. 1C), we pursued RAB35 because, in addition to meeting all of the criteria mentioned previously, it is a well-studied GTPase for which reagents and tools are readily available. RAB35 is a regulator of cytoskeletal organization and trafficking at the recycling endosome (8–11). Endomembrane trafficking by RAB35 is regulated by the DENND1 family of RAB35 guanine nucleotide exchange factors (9, 12–14) and by the TBC1D10 family of RAB35 GTPase activating proteins (15–17). Because RAB35 was
a robust hit in our screen (fig. S1A), has not been implicated as a regulator of PI3K/AKT signaling, and has somatic mutations that have been reported in human cancers mapping to residues conserved in other small GTPases (Fig. 1D and Table S7), we prioritized RAB35 for further study.

Next, we confirmed that depletion of RAB35 with the same shRNAs that scored in the original screen suppressed AKT phosphorylation to a similar extent as depletion of the mTORC2 component RICTOR (Fig. 1E). Furthermore, additional shRAB35 hairpins not present in the original screen also suppressed AKT phosphorylation, as well as the AKT substrate FOXO1/3A (Fig. S1B). Thus, multiple independent shRNAs targeting RAB35 consistently impair AKT phosphorylation in HeLa cells.

To ensure that the effect of RAB35 knockdown on AKT signaling was not cell-type specific, we depleted RAB35 protein in human embryonic kidney (HEK) 293E cells and in murine NIH-3T3 cells (Fig. 2, A and B). Knockdowns of RAB35 with two different shRNAs diminished AKT phosphorylation at S473 in response to serum stimulation in both human and mouse cells and did so more potently than RICTOR. Furthermore, the PI3K-dependent phosphorylation of FOXO1/3A and the SGK1 substrate NDRG-1 was also decreased in cells depleted of RAB35. We expanded this analysis to seven additional cancer cell lines with different mutational backgrounds (oncogenic PI3KCA or KRAS, deleted PTEN, mutated NF2 or TP53) and generally found reduced AKT phosphorylation after RAB35 depletion, with some modest differences that are likely attributable to distinct hairpins in distinct cellular contexts (fig. S2, A to C). Furthermore, AKT phosphorylation at the PDK1 phosphorylation site T308 was consistently reduced by RAB35 knockdown, suggesting that RAB35 may function upstream of PDK1. Collectively, these data indicate that RAB35 is broadly necessary for the efficient activation of PI3K/AKT signaling in response to serum stimulation.

To explore whether RAB35 is sufficient to activate the PI3K/AKT pathway, we generated cell lines that stably expressed either wild-type RAB35 (RAB35wt) or the dominant active GTPase-deficient, guanosine triphosphate (GTP)–bound RAB35 Q67L (RAB35Q67L). Cells expressing the wild-type RHEB GTPase (RHEBwt), which regulates mTORC1, served as a control. Stable expression of either RHEBwt or RAB35Q67L did not alter phosphorylation of AKT or extra-cellular signal–regulated kinase (ERK) in response to growth factor signaling (Fig. 2C and Fig. S3). However, expression of active RAB35Q67L mutant rendered AKT phosphorylation (at both T308 and S473) constitutively elevated and refractory to growth factor deprivation but did not affect ERK phosphorylation. FOXO1/3A phosphorylation was also consistently elevated in serum-deprived cells expressing RAB35Q67L. Therefore, the expression of GTP-bound RAB35 is sufficient to activate PI3K/AKT signaling in cells, even in the absence of growth factors.

The fact that RAB35Q67L results in constitutive phosphorylation of T308 as well as S473 suggests that RAB35 may function upstream of the
T308 kinase PDK1 (18, 19). However, attribution of RAB35 function to PDK1 versus mTORC2 activation is challenging because the phosphorylation states of T308 and S473 are interdependent (20–22). To address this issue, we took advantage of cells that stably express alleles of murine AKT1 (mAkt1) in which either T308 or S473 were mutated to phosphomimetic aspartate residues (23) (fig. S4, A and B). As expected, mTOR blockade with the TORC1/2 inhibitor torin1 or blockade by RICTOR depletion (resulting in loss of mTORC2 function) suppressed phosphorylation at both sites in mAkt1S473D-expressing cells (fig. S4C). In mAkt1T308D-expressing cells, which lack a regulated mTORC2 site, these same interventions either had no effect on T308 phosphorylation (RICTOR depletion) or enhanced T308 phosphorylation (torin1).

Having confirmed the ability of this system to distinguish direct effects on T308 versus S473, we examined the impact of RAB35 knockdown. Unlike depletion of RICTOR or treatment with torin1, depletion of RAB35 decreased T308 phosphorylation in mAkt1wt- and mAkt1S473D-expressing cells. RAB35 depletion also decreased S473 phosphorylation in mAkt1wt- and mAktT308D-expressing cells (fig. S4C). These data suggest that RAB35 signals to AKT by functioning upstream of both PDK1 and mTORC2.

We reasoned that if RAB35 acts upstream of both PDK1 and mTORC2, it may be controlling their common regulator, PI3K. Although we could not detect an interaction between endogenous RAB35 and PI3K, we found that stably expressed RAB35, but not RHEB, copurified with PI3K when the kinase was immunoprecipitated by its p85 subunit (Fig. 2D). Interestingly, more FLAG-tagged RAB35 (FLAG-RAB35) copurified with PI3K in immunoprecipitates from cells expressing constitutively GTP-bound RAB35G12V than from cells expressing RAB35WT. Conversely, we found that the same experiment performed using lysates from cells stably expressing a constitutively guanosine diphosphate–bound allele of RAB35 (RAB35S22N) did not recover any FLAG-RAB35. Thus, the physical association of RAB35 with PI3K is nucleotide-dependent. We also asked whether RAB35 is necessary for PI3K kinase activity in vitro. As expected, PI3K immunopurified from cells depleted of RAB35 had a ~50% reduction in PI3K kinase activity in vitro. Further, we found that depletion of RAB35 did not reduce the amount of p110α recovered in p85 immunoprecipitates, which suggests that RAB35 depletion does not disrupt the p85-p110α interaction (fig. S5B). Thus, RAB35 copurifies with PI3K in a nucleotide-dependent manner and may be required for optimal PI3K enzymatic function.

Given that RAB35 functions upstream of PI3K, we investigated whether RAB35 acts downstream of any particular growth factor receptor. Depletion of RICTOR, PI3Kα, or RAB35 in HEK293 cells reduced S473 phosphorylation of AKT in response to insulin stimulation. We next asked whether RAB35 is necessary for PI3K activity in a PI3K-dependent context. NIH-3T3 cells stably expressing the indicated RAB35 alleles, HA-p110αΔT2045R, or myristoylated FLAG-AKT1WT were cultured for 4 weeks in medium containing 2% BCS with either dimethyl sulfoxide (DMSO) or 250 nM GDC-0941 (a pan-PI3K inhibitor), then fixed, stained with crystal violet, and imaged.

**Fig. 3.** RAB35 mutants identified in human tumors activate PI3K signaling, suppress apoptosis, and are transforming in vitro. (A) Two mutations in RAB35 that code for amino acid changes A151T and F161L were identified in the MSKCC Cbio and COSMIC data sets. Alignment of RAB35 with KRAS was performed using ClustalW2. (B) Mutant RAB35 alleles from human tumors can activate PI3K/AKT signaling. (C) NIH-3T3 cells stably expressing the indicated proteins were plated and treated as indicated for 4 hours with or without serum-containing medium. Cells were then lysed, and the cell lysates were immunoblotted for the indicated proteins. (D) NIH-3T3 cells were treated as in (C) and trypsinized, and the number of viable cells was counted. Cell counts were normalized to the number of viable cells for each cell line from non–serum-starved conditions. Graph bars represent mean normalized cell count; error bars indicate SD (n = 3 replicates); and asterisks indicate statistical significance (P < 0.05), as determined by a Student’s t test. (E) RAB35 mutants can transform cells in vitro in a PI3K-dependent manner. NIH-3T3 cells stably expressing the indicated RAB35 alleles, HA-p110αΔT2045R, or myristoylated FLAG-AKT1WT were cultured for 4 weeks in medium containing 2% BCS with either dimethyl sulfoxide (DMSO) or 250 nM GDC-0941 (a pan-PI3K inhibitor), then fixed, stained with crystal violet, and imaged.
to treatment with either insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), platelet-derived growth factor AA (PDGF-AA), or vascular endothelial growth factor (VEGF) (fig. S6A). RAB35 depletion did not dramatically alter phosphorylation of ERK or tyrosine phosphorylation of receptors for IGF, EGF, PDGF, or VEGF (IGFR, EGFR, PDGFR, or VEGFR, respectively) (fig. S6, B and C). Collectively, these data suggest a model in which RAB35 functions upstream of PI3K and downstream of growth factor RTKs to activate AKT.

One criterion for prioritization of genes on our hit list (table S6) was evidence for mutation in human cancer databases (table S7). For RAB35, missense mutations have been reported at two residues that are conserved in RAS-like GTPases, A151T and F161L. We noticed that the A151T and F161L mutations in RAB35 were markedly similar to two well-documented KRAS mutations (A146T and F156L) that have been previously identified in human tumor samples (Fig. 3A) (24, 25). Although they are not canonical activating mutations, stable expression of either of these two KRAS mutants was sufficient to activate ERK signaling and transform NIH-3T3 cells in vitro. We therefore asked whether the two similar mutations in RAB35 might also be gain-of-function mutations that activate RAB35 signaling.

To test this possibility, we generated NIH-3T3 cells stably expressing either RAB35wt, GTPase-deficient RAB35Q67L, or the RAB35A151T and RAB35F161L alleles reported in human tumors. As expected from our earlier experiments in human cells, expression of RAB35Q67L did not activate AKT phosphorylation upon serum deprivation, whereas expression of the GTPase-deficient RAB35Q67L did (Fig. 3B). Notably, expression of the two naturally occurring RAB35A151T and RAB35F161L mutants also elevated AKT phosphorylation levels during serum deprivation, indicating that both are gain-of-function alleles sufficient to activate PI3K/AKT signaling.

Because PI3K/AKT signaling inhibits apoptosis, we next examined whether cells expressing mutant RAB35 alleles are resistant to apoptosis triggered by growth factor withdrawal. Four hours of serum deprivation of NIH-3T3 cells stably

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**Fig. 4. Expression of dominant active RAB35\textsuperscript{Q67L} activates PI3K signaling via PDGFR\textalpha.** (A) Stable expression of dominant active RAB35\textsuperscript{Q67L} activates the PDGFR. HEK-293E cells stably expressing the indicated FLAG-tagged GTPases were serum-starved, treated as indicated, lysed, and analyzed by immunoblotting. (B) Pharmacologic inhibition of PDGFR\textalpha inhibits PI3K/AKT activity in cells expressing dominant active RAB35\textsuperscript{Q67L}. Cells stably expressing RAB35\textsuperscript{Q67L} were treated with DMSO, a pan-PI3K inhibitor (PI3Ki) (GDC-0941, 0.5 μM), a PI3K\alpha-specific inhibitor (PI3K\alphai) (BLY719, 1 μM), a PDGFR\beta inhibitor (CP-673451, 1 μM), or a PDGFR\beta inhibitor (creolanib, CP-868596, 1 μM) in serum-free media overnight then lysed and analyzed as in (A). (C) PDGFR\alpha is trafficked to LAMP2-positive endomembranes after stimulation with PDGF. HEK-293E cells stably expressing FLAG-RAB35\textsuperscript{Q67L} were transfected with cDNA expressing PDGFR\alpha-myc, serum-starved and then treated with PDGF-AA (100 ng/ml) for 15 min, and processed for immunofluorescence with anti-myc and anti-LAMP2 antibodies. (D) Expression of RAB35\textsuperscript{Q67L} traffics PDGFR\alpha to LAMP2-positive endomembranes in the absence of PDGF. HEK-293E cells stably expressing FLAG-RAB35\textsuperscript{Q67L} were transfected with cDNA coding for PDGFR\alpha-myc and then treated and processed for immunofluorescence as in (C). Scale bars in (C) and (D) represent 10 μm. (E) Cartoon depicting the trafficking of PDGFR\alpha to LAMP2-positive endomembranes in cells expressing RAB35\textsuperscript{Q67L}. In cells expressing RAB35\textsuperscript{Q67L}, PDGFR\alpha is activated at the cell membrane by PDGF-AA ligand and then internalized to EEA1-positive and LAMP2-positive endomembranes, where liganded PDGFR\alpha drives PI3K/AKT signaling. Dashed lines reflect the fact that endomembrane-based signaling may not be as prominent as plasma membrane signaling in normal states (left). In cells expressing GTPase-deficient, GTP-bound RAB35\textsuperscript{Q67L}, unliganded PDGFR\alpha is internalized to EEA1- and LAMP2-positive compartments, where it drives constitutive activation of PI3K/AKT signaling (right).
expressing RAB35WT were sufficient to elevate cleaved levels of the apoptotic markers PARP and caspase 3 (Fig. 3C). In comparison, cells stably expressing one of the three RAB35 mutants or oncogenic p110αH1047R had decreased levels of cleaved PARP and cleaved caspase 3 after serum withdrawal. Further, although cells expressing RAB35 WT died in response to serum deprivation, cells expressing mutant alleles of RAB35 or p110αH1047R had significantly improved cell viability when deprived of growth factors (Fig. 3D). Taken together, these data suggest that mutant RAB35 proteins can mitigate cell death in response to growth factor deprivation.

To determine whether the mutant alleles of RAB35 are oncogenic, we examined their activity in a standard NIH-3T3 cell focus-formation assay—an in vitro model of density-independent growth that often correlates with tumorigenicity (26). NIH-3T3 cells expressing oncogenic p110αH1047R myristoylated AKT1 (AKT1myr), or any of the three mutant alleles of RAB35, but not RAB35 WT, formed foci (Fig. 3E and fig. S7). Moreover, we found that when cultured in medium containing 250 nM of the pan-class I PI3K inhibitor GDC-0941 (27), cells expressing RAB35 mutants and p110αH1047R were unable to form foci. Not surprisingly, PI3K blockade did not inhibit the growth of cells transduced with AKT1myr. Thus, the expression of the RAB35 mutants identified in human cancers can transform NIH-3T3 cells in vitro in a PI3K-dependent manner.

Because RAB proteins regulate endomembrane trafficking, we considered whether our evidence implicating RAB35 in AKT activation, downstream of RTKs and upstream of PI3K, is linked to this more established function of RAB proteins. The fact that many RTKs undergo ligand-stimulated internalization and recycling through endosomes, while remaining competent to signal (28–32), suggests that RAB35 may function in this process. In considering this possibility, we noted highly elevated levels of tyrosine phosphorylation of the PDGFRα/β—but not of IGF1R, fibroblast growth factor receptor, VEGFR, or MET—in cells expressing the constitutively active RAB35Q67L allele (Fig. 4A and fig. S8). This increase in PDGFRα/β phosphorylation was markedly reduced by treatment of cells with crenolanib (CP-868596), a PDGFR inhibitor that affects both PDGFRα and PDGFRβ with similar potency (33–35) (Fig. 4B and fig. S8). Notably, crenolanib treatment also diminished phosphorylation of AKT and the downstream substrate FOXO3A, suggesting that the RAB35Q67L allele activates AKT through PDGFR. Treatment with a more β-selective PDGFR inhibitor (CP-673451) only moderately affected AKT phosphorylation, implicating PDGFRα as the most likely RAB35Q67L target. The pan-PI3K inhibitor (GDC-0941) and the PI3Kα-specific inhibitor (BLY719) both suppressed AKT phosphorylation but not PDGFRα/β phosphorylation, providing further evidence that PDGFR/PDAB35Q67L functions upstream of PI3K.

We postulated that RAB35Q67L might initiate PDGFR-mediated AKT activation by altering PDGFRα localization. To explore this possibility, we transfected cells that stably expressed either RAB35WT or RAB35Q67L with cDNA encoding for myc-tagged PDGFRα and studied the localization of tagged PDGFRα in the presence or absence of the PDGFRα ligand PDGF-AA (fig. S10). As expected, in cells that expressed RAB35WT, stimulation with PDGF-AA relocalized PDGFRα into punctate intracellular structures. Notably, PDGFRα was constitutively localized to punctate structures in RAB35Q67L-expressing cells, even in the absence of PDGF-AA stimulation. This effect was specific to PDGFRα, because internalization of EGFR was not altered by RAB35Q67L (fig. S11). By simultaneously visualizing PDGFRα-myc with the lysosomal protein LAMP2 or the early endosome marker EEA1, we next explored whether the punctate localization of PDGFRα in RAB35Q67L-expressing cells was the same as that seen in the normal physiologic context of PDGFRα activation by PDGF-AA. In both PDGF-AA-stimulated RAB35WT cells and unstimulated RAB35Q67L-expressing cells, transfected PDGFRα-myc colocalized with endogenous LAMP2 (Fig. 4, C and D, and fig. S12) and EEA1 (fig. S13). We also found that FLAG-RAB35 is present in endomembranes that contain LAMP2 (fig. S14A) but not in those that contain EEA1 (fig. S14B). This suggests that PI3K activity downstream of RAB35Q67L may originate on LAMP2-containing endosomes, in a pattern that mirrors that seen with native PDGF-AA ligand, is consistent with this model. Alternatively, these mutations may be neomorphic, conferring a novel molecular function to RAB35 that results in signaling in a RAS-like manner. The fact that other RAB proteins (RAB1B, RAB39, RAB13, RASEF) emerged as hits in our screen and have rare missense mutations reported in human tumors (table S6) begs a broader examination of this question.

REFERENCES AND NOTES

4. Single-letter abbreviations for the amino acid residues are as follows: A; Ala; C; Cys; D; Asp; E; Glu; F; Phe; G; Gly; H; His; I; Ile; K; Lys; L; Leu; M; Met; A; Asp; P; Pro; Q; G; Gln; R; Arg; S; Ser; T; Thr; V; Val; W; Trp; and Y; Tyr.
5. See supplementary materials and methods for a detailed explanation of z score calculation and hit criteria.
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Dengue subgenomic RNA binds TRIM25 to inhibit interferon expression for epidemiological fitness

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The global spread of dengue virus (DENV) infections has increased viral genetic diversity, some of which appears associated with greater epidemiological potential. The mechanisms governing viral fitness in epidemiological settings, however, remain poorly defined. We identified a determinant of fitness in a foreign dominant (PR-2B) DENV serotype 2 (DENV-2) clade, which emerged during the 1994 epidemic in Puerto Rico and replaced an endemic (PR-1) DENV-2 clade. The PR-2B DENV-2 produced increased levels of subgenomic flavivirus RNA (sfRNA) relative to genomic RNA during replication. PR-2B sfRNA showed sequence-dependent binding to and prevention of tripartite motif 25 (TRIM25) deubiquitylation, which is critical for sustained and amplified retinoic acid–inducible gene 1 (RIG-I)–induced type I interferon expression. Our findings demonstrate a distinctive viral RNA–host protein interaction to evade the innate immune response for increased epidemiological fitness.

To gain insights into how DENV genome variation affects its epidemiological phenotype, we examined a DENV-2 clade replacement event that coincided with an epidemic of severe dengue in Puerto Rico in 1994 (1, 7, 8). Phylogenetic analysis of the complete coding sequences of DENV-2 isolated from Puerto Rico and neighboring countries showed that three distinct clades within the Asian/American genotype coexisted from Puerto Rico and neighboring countries and were predominant in the epidemic clade of viruses is not specific to PR-1 viruses (Fig. 1D). While the absolute values of the sfRNA ratios in PR-2B as compared to PR-1 viruses (Fig. 1D). While the absolute values of the sfRNA levels between PR-1 and PR-2B viruses was smaller, although statistically significant (Fig. 1C), resulting in higher sfRNA:grNA ratios in PR-2B than PR-1 viruses (Fig. 1D).}

**VIROLOGY**

**Dengue subgenomic RNA binds TRIM25 to inhibit interferon expression for epidemiological fitness**

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Identification of an oncogenic RAB protein
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Science 350, 211 (2015);
DOI: 10.1126/science.aaa4903

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