Small-molecule hydrophobic tagging–induced degradation of HaloTag fusion proteins

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The ability to regulate any protein of interest in living systems with small molecules remains a challenge. We hypothesized that appending a hydrophobic moiety to the surface of a protein would mimic the partially denatured state of the protein, thus engaging the cellular quality control machinery to induce its proteasomal degradation. We designed and synthesized bifunctional small molecules to bind a bacterial dehalogenase (the HaloTag protein) and present a hydrophobic group on its surface. Hydrophobic tagging of the HaloTag protein with an adamantyl moiety induced the degradation of cytosolic, isoprenylated and transmembrane HaloTag fusion proteins in cell culture. We demonstrated the in vivo utility of hydrophobic tagging by degrading proteins expressed in zebrafish embryos and by inhibiting HrasG12V–driven tumor progression in mice. Therefore, hydrophobic tagging of HaloTag fusion proteins affords small-molecule control over any protein of interest, making it an ideal system for validating potential drug targets in disease models.

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Supplementary Results

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cation high stability and cell permeability of compounds bearing de we continued our investigation of hydrophobic tagging–induced the luciferase activity (HyT5 control compound with two PEG groups did not decrease and were active in a concentration-dependent manner, whereas the HyTs showed high hydrophobicity scores (logP ranging from 3 to 5) characterized the five most potent compounds further (Fig. 1a). All five HyTs showed high hydrophobicity scores (logP ranging from 3 to 5) and were active in a concentration-dependent manner, whereas the HyT5 control compound with two PEG groups did not decrease the luciferase activity (Fig. 1b). On the basis of the initial data, we continued our investigation of hydrophobic tagging–induced degradation with the hydrophobic compound HyT13 because of the reported high stability and cell permeability of compounds bearing adamantyl groups. As our luciferase assay relied on the loss of activity of the luciferase–HaloTag fusion protein, we wanted to determine whether the decrease in luciferase activity resulted from the degradation of the entire fusion protein or simply from the inhibition of luciferase activity. We generated a stable HEK 293T-based cell line with a single integration site containing a fusion protein of hemagglutinin (HA), enhanced green fluorescent protein (EGFP) and HaloTag (HA–EGFP–HaloTag) and used this cell line to perform kinetic studies with HyT13. Immunoblotting showed that HyT13 efficiently degraded the fusion protein, achieving its maximal effect at 100 nM (Fig. 2a). The half-maximum inhibitory concentration (IC50) of HyT13 was determined to be 21 nM (Supplementary Results, Supplementary Fig. 1). A time-course experiment revealed that full inhibition was reached within 8 h, with 50% degradation observed by 1.5 h (Fig. 2b and Supplementary Fig. 2). When cells were treated with 1 μM HyT13 for 24 h and then removed from the HyT13 for 24 h, the protein level recovered to half the starting levels. No cellular toxicity was observed at 20 μM HyT13, a dose 1,000-fold the IC50 value (Supplementary Fig. 3). Consistent with our hypothesis that hydrophobic tagging mimics a partially denatured protein state and that the protein is ultimately delivered to the proteasome for degradation, inclusion of the proteasome inhibitors MG132 and YU101 (ref. 29) blocked HyT13-mediated degradation (Fig. 2c). To verify that the observed decrease in HA–EGFP–HaloTag abundance did not result from masking of the HA epitope during immunoblotting, we generated a HeLa cell line stably expressing EGFP–HaloTag and analyzed the intracellular fluorescence by flow cytometry. Consistent with our previous observations, treatment of these cells with 1 μM HyT13 for 24 h reduced the mean fluorescence intensity of cells by 84% (Fig. 2d). Together, these findings provide experimental evidence that hydrophobic tagging represents a viable strategy for the control of protein levels.

Degradation of transmembrane and zebrafish proteins

Existing technologies for small molecule–mediated control of protein levels are limited by the challenge of degrading transmembrane proteins. To determine whether hydrophobic tagging shares this limitation, we constructed several transmembrane HA–HaloTag fusion proteins such that the HaloTag portion would be intracellular. Ror2 is a single-pass receptor tyrosine kinase–like orphan receptor, which functions in Wnt ligand signaling.

RESULTS

Hydrophobic tagging destabilizes HaloTag fusion proteins

We designed 21 structurally distinct scaffolds as the basis for our hydrophobic tags (HyTs). Across these scaffolds, we tested 30 compounds, synthesized by fusing hydrophobic moieties to the HaloTag haloalkane reactive linker (Supplementary Table 1). In designing the hydrophobic portion of these bifunctional molecules, we used the compound library available in the Yale University Small Molecule Discovery Center as an informal resource to identify compounds that maximized hydrophobicity, minimized molecular weight and incorporated chemically diverse and commercially available scaffolds. To determine their biological activity, we designed 21 structurally distinct scaffolds as the basis for our hydrophobic tagging–induced degradation with the hydrophobic compound HyT13 because of the reported high stability and cell permeability of compounds bearing adamantyl groups.

As our luciferase assay relied on the loss of activity of the luciferase–HaloTag fusion protein, we wanted to determine whether the decrease in luciferase activity resulted from the degradation of the entire fusion protein or simply from the inhibition of luciferase activity. We generated a stable HEK 293T-based cell line with a single integration site containing a fusion protein of hemagglutinin (HA), enhanced green fluorescent protein (EGFP) and HaloTag (HA–EGFP–HaloTag) and used this cell line to perform kinetic studies with HyT13. Immunoblotting showed that HyT13 efficiently degraded the fusion protein, achieving its maximal effect at 100 nM (Fig. 2a). The half-maximum inhibitory concentration (IC50) of HyT13 was determined to be 21 nM (Supplementary Results, Supplementary Fig. 1). A time-course experiment revealed that full inhibition was reached within 8 h, with 50% degradation observed by 1.5 h (Fig. 2b and Supplementary Fig. 2). When cells were treated with 1 μM HyT13 for 24 h and then removed from the HyT13 for 24 h, the protein level recovered to half the starting levels. No cellular toxicity was observed at 20 μM HyT13, a dose 1,000-fold the IC50 value (Supplementary Fig. 3). Consistent with our hypothesis that hydrophobic tagging mimics a partially denatured protein state and that the protein is ultimately delivered to the proteasome for degradation, inclusion of the proteasome inhibitors MG132 and YU101 (ref. 29) blocked HyT13-mediated degradation (Fig. 2c). To verify that the observed decrease in HA–EGFP–HaloTag abundance did not result from masking of the HA epitope during immunoblotting, we generated a HeLa cell line stably expressing EGFP–HaloTag and analyzed the intracellular fluorescence by flow cytometry. Consistent with our previous observations, treatment of these cells with 1 μM HyT13 for 24 h reduced the mean fluorescence intensity of cells by 84% (Fig. 2d). Together, these findings provide experimental evidence that hydrophobic tagging represents a viable strategy for the control of protein levels.

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![Figure 1](https://example.com/figure1.png)

**Figure 1** | Hydrophobic tagging strategy using the HaloTag fusion protein system. (a) Chemical structures of the representative HaloTag ligands: HyT5 (1), HyT12 (2), HyT13 (3), HyT16 (4), HyT21 (5) and HyT22 (6). (b) HEK 293T cells expressing HA–HaloTag–luciferase were treated with the indicated compounds at 1 μM for 24 h, at which point luciferase assays were performed. Error bars, s.e.m.
Figure 2 | HyT13 leads to degradation of HaloTag fusion proteins. (a) FliP-In 293 cells expressing HA–EGFP–HaloTag were treated with the indicated concentrations of HyT13 for 24 h. The lysates were probed with HA-specific and β-actin-specific antibodies. (b) The same cell line as in (a) was treated for the indicated times with 1 μM HyT13. The rightmost sample was treated with HyT13 for 24 h, after which HyT13-free medium was provided for 24 h. (c) The same cell line as in (a) was pretreated with proteasome inhibitors MG132 (10 μM) and YU01 (10 μM) for 1 h before addition of 1 μM HyT13. The lysates were prepared from cells 6 h after HyT13 addition. (d) HeLa cells stably expressing EGFP–HaloTag were treated with vehicle or 1 μM HyT13 for 24 h, whereupon the intracellular GFP fluorescence was quantified by flow cytometry. (e) HEK 293T cells stably expressing the indicated transmembrane HaloTag–Hras1 fusion proteins were treated with 1 μM HyT13 for 24 h. Shown are representative images from at least three experiments; bands were quantified, and the mean degradation ± s.e.m. is shown. (f) Zebrafish embryos were injected with HA–HaloTag–Smad5 cRNA and treated with 10 μM HyT13 for 24 h. Shown are representative images from at least three experiments; bands were quantified and mean degradation ± s.e.m. is shown. Full gels are available in the Supplementary Results.

Likewise, CD3E is a single-pass cell surface glycoprotein involved in antigen recognition19 and CD9 is a four-pass transmembrane protein from the tetraspanin family that functions in integrin signaling24. Similarly, the G protein–coupled receptors GPR40 and Frizzled-4 are seven-pass transmembrane receptors for long-chain free fatty acids and Wnt proteins, respectively13,24. Treatment of HEK 293T cell lines stably expressing these transmembrane HaloTag fusion proteins with HyT13 efficiently induced their degradation (Fig. 2e), demonstrating the potential of our hydrophobic tagging system to degrade transmembrane proteins. These experiments show that fusions to either the N or C terminus of the HaloTag protein are susceptible to our small molecule–induced degradation strategy and that transmembrane proteins can be degraded by HyT13.

We also explored the possibility of using the hydrophobic tagging system in the zebrafish Danio rerio. We injected HA–HaloTag–Smad5 cRNA into zebrafish embryos and then treated the embryos with either vehicle (DMSO) or HyT13. Immunoblotting of injected embryo lysates revealed that the fusion protein was very efficiently degraded, indicating that HyT13 is able to penetrate the chorion and directly degrade the HaloTag fusion proteins for degradation in zebrafish (Fig. 2f). These experiments show that HyT13 is capable of degrading fusion proteins in various cell lines as well as in zebrafish embryos.

**Figure 3b**

**G12V**

We injected HA–EGFP–HaloTag fusion proteins into the tetrascernin family that functions in integrin signaling19. Similarly, the G protein–coupled receptors GPR40 and Frizzled-4 are seven-pass transmembrane receptors for long-chain free fatty acids and Wnt proteins, respectively13,24. Treatment of HEK 293T cell lines stably expressing these transmembrane HaloTag fusion proteins with HyT13 efficiently induced their degradation (Fig. 2e), demonstrating the potential of our hydrophobic tagging system to degrade transmembrane proteins. These experiments show that fusions to either the N or C terminus of the HaloTag protein are susceptible to our small molecule–induced degradation strategy and that transmembrane proteins can be degraded by HyT13.

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**HyT13 suppresses HaloTag-Hras1<sup>G12V</sup> tumor burden in mice**

We explored the functional utility of HaloTag-based degradation of an oncogene by HyT13 both in cell culture and in mice. The human gene HRAS1, which encodes a small GTPase, is one of the most commonly mutated genes in cancer, with up to 90% of cancers harboring activating mutations in this gene<sup>36</sup>. Activating mutations, such as the HRAS1<sup>G12V</sup> allele, lead to decreased dependence on extracellular mitogenic signals. Ectopic expression of HRAS1<sup>G12V</sup> in the mouse fibroblast cell line NIH-3T3 can lead to a transformed phenotype, as demonstrated by assays in cell culture and mice<sup>36,37</sup>. When Hras1<sup>G12V</sup>-expressing cells are grown in culture under low-serum conditions, they lose cell-to-cell contact inhibition and form distinct foci instead of growing as a cellular monolayer. Furthermore, these transformed cells are capable of tumor formation when injected into immunocompromised nude mice<sup>36,37</sup>. We investigated whether HaloTag–Hras1<sup>G12V</sup>–driven focus formation could be suppressed in NIH-3T3 cells and whether the HaloTag–Hras1<sup>G12V</sup>–driven tumor burden in mice could be reduced by administration of HyT13. First, NIH-3T3 cells were stably infected with a retroviral construct encoding HA–HaloTag–Hras1<sup>G12V</sup>. The fusion protein was readily degraded with HyT13 (Fig. 3a). To test the HaloTag receptor specificity for HyT13, we generated a point mutation in the gene encoding HaloTag, yielding a HaloTag protein (HaloTag<sub>0124</sub>) that is unable to form a covalent bond with the reactive chloroalkane in HyT13 (ref. 26). Unlike HA–HaloTag–Hras1<sup>G12V</sup>, the HA–HaloTag<sub>0124</sub>–Hras1<sup>G12V</sup> fusion protein was unaffected by HyT13 (Fig. 3a). Next, we plated both cell lines sparsely (1 × 10<sup>4</sup> cells per 10-cm<sup>2</sup> plate) in 10% FBS–containing medium. The next day, we replaced the medium with 1% FBS–containing medium and treated the cultures with either vehicle or HyT13. By day 6, both vehicle-treated cell lines and HyT13-treated, HA–HaloTag<sub>0124</sub>–Hras1<sup>G12V</sup>-expressing cells had formed many foci, whereas HA–HaloTag–Hras1<sup>G12V</sup>-expressing cells treated with HyT13 had grown a normal monolayer of cells, much like the parental NIH-3T3 cells (Fig. 3b,c). In the absence of HyT13, HA–HaloTag–Hras1<sup>G12V</sup>-expressing cells yielded a slightly larger number of colonies than HA–HaloTag<sub>0124</sub>–Hras1<sup>G12V</sup> cells as well (data not shown). These results indicate that hydrophobic tagging can be used to reduce protein activity in <i>vitro</i> cell culture.

To examine whether the HaloTag–HyT13-based system could be used in mouse models to relieve the Hras1<sup>G12V</sup>–driven tumor burden, we first evaluated the pharmacokinetics of HyT13. We performed a maximum-tolerated-dose experiment with HyT13 in nude mice at doses up to 100 mg per kg body weight over a 14-d
Figure 3 | Functional validation of HaloTag degradation by HyT13. (a) NIH-3T3 cells expressing either HA-HaloTag–Hras1G12V or HA–HaloTagD106A–Hras1G12V were treated with vehicle or 1 μM HyT13 for 24 h. The lysates were prepared for immunoblotting, and the blots were probed with HA-specific and β-actin–specific antibodies. Full gels are available in the Supplementary Results. (b) Focus formation assay was performed with NIH-3T3 cells infected with HA–HaloTag–Hras1G12V or HA–HaloTagD106A–Hras1G12V. The cells were treated with vehicle or 1 μM HyT13. Scale bars, 5 mm. (c) Quantification of foci as described in b. The number of foci per cm² was counted from three separate plates. Error bars, s.e.m. (d) HA–HaloTag–Hras1G12V-expressing NIH-3T3 cells were injected into the flank of nude mice on day 0. The mice were administered i.p. injections of vehicle or HyT13 daily from day 0. Error bars, s.e.m. (n = 7 for each treatment group).

DISCUSSION

Here we describe a new hydrophobic tagging technology that can systematically degrade a specific protein upon addition of a small molecule (Fig. 4). This strategy has several advantages over the existing technologies. First, protein degradation is achieved upon compound administration as opposed to after ligand withdrawal. This is particularly relevant when a protein needs to be expressed for long periods before the study, as continuous ligand treatment is not necessary to maintain expression of the POI. In contrast, destabilizing domain–based methods of controlling protein abundance require constant drug administration, which can be time consuming and expensive. Also, the concentration of the fusion protein between ligand administrations is likely to fluctuate in the destabilizing domain–based system, whereas expression of the HaloTag fusion protein is stable in the absence of the degradation signal. Therefore, depending on the application, it can be desirable to have a system where the small molecule induces degradation, not stabilization, of the POI. Second, our HaloTag–HyT13 method relies on the introduction of a single fusion domain to the POI. This feature contrasts with the auxin system, where an exogenous plant E3 ligase must be expressed in addition to the fusion protein. Third, almost all human and mouse genes are commercially available as both N- and C-terminal HaloTag fusions in transient and lentiviral expression vectors. Proteins fused to the 34-kDa HaloTag receptor are proving useful in many studies of protein function, as they can be readily labeled in vivo and purified with fluorescent or biotinylated HaloTag reagents. The ability to degrade these fusion proteins with the hydrophobic tag HyT13 is an important addition to the repertoire of possible HaloTag applications. Although HyT13 is not yet commercially available, this small molecule can be obtained with standard synthetic methods in four steps from commercially available starting materials with an overall yield of 63% (Supplementary Methods, Supplementary Scheme 2).

One of the criticisms that surround the FKBP12-based degradation systems is their reliance on rapamycin, FK506 or their derivatives to cause protein perturbation. As these are bioactive small molecules, they could induce biological effects unrelated to POI perturbation. In contrast, HaloTag dehalogenase is a bacterial protein, and covalent binding of HyT13 to HaloTag affords this system a high degree of specificity. This bioorthogonality may explain the lack of noticeable HyT13 cytotoxicity, even upon administration of 1,000 times its IC₅₀ value of 21 nM in cell culture. Moreover, mice injected daily with HyT13 at 100 mg per kg body weight for 14 d gained weight normally, suggesting that HyT13 possesses no in vivo toxicity even at this high dose.
Like several other systematic degradation methods, the HaloTag–HyT13 methodology is not able to degrade endogenous proteins in culture or in live animals unless the HaloTag-encoding gene is fused with the gene of interest. There are, however, two viable strategies to overcome this limitation. First, it is possible to generate HaloTag fusion constructs via targeted genome engineering. Recent advances in zinc finger nucleases\textsuperscript{38,39} and homologous recombination\textsuperscript{40} technologies have opened up the possibility of systematically tagging endogenous proteins in rodents in a manner similar to yeast. The second approach would be to inactivate the endogenous gene by knockdown or knockout techniques and introduce the corresponding HaloTag fusion gene into the animal. Both approaches should also make it possible to bypass an early requirement for a particular essential gene during development, thus allowing its function to be studied later during organogenesis or disease development.

In summary, we describe a chemical biology approach to systematically degrade any POI in either cell culture or whole animals. The system requires the construction of a single fusion protein, which is specifically degraded by the addition of a nontoxic, low-molecular-weight hydrophobic tag. We believe this system is particularly amenable to animal studies, as we have shown here with experiments in zebrafish and mice. Our findings also suggest that hydrophobic tagging is a unique approach to promote targeted degradation of endogenous proteins independent of the HaloTag–HyT13 system.

METHODS

Synthesis and characterization of HyT compounds is described in the Supplementary Methods.

Cell culture and materials. HEK 293 cells used in this study were FRT recombination-based 293 Flp-In cells (Invitrogen), along with HeLa and NIH-3T3 cells. All cells were grown at 37 °C in DMEM and supplemented with 10% FBS, 100 U ml\textsuperscript{−1} penicillin and 100 μg ml\textsuperscript{−1} streptomycin. The HaloTag protein was obtained from pH2T vector (Promega), and the luciferase sequence was obtained from pGL3-Basic vector (Promega). S. Stricker provided mouse Ror2, and G. Daley provided the retroviral pEYK.1 vector. Danio rerio HaloTag Smad5 was cloned from a zebrafish cdna library, HRAS\textsuperscript{G12V} was obtained from Addgene plasmid 9051 (provided by R. Weinberg), and the remaining transmembrane proteins were cloned from a human spleen cDNA library (Invitrogen). A D106A point mutation was introduced into the gene encoding HaloTag by the QuikChange Site Directed mutagenesis kit (Stratagene). HA–HaloTag–Smad5 and EGFP–HaloTag were cloned into the pCS2+ vector, whereas all other constructs were cloned into a retroviral pEYK.1 vector by excising GFP\textsuperscript{41}. Retrovirus was generated in GP2–293 cells (Clontech) with a pSVG-G vesicular stomatitis virus retroviral vector and a corresponding pEYK plasmid, and the cells indicated in Figure 3b were infected as previously described\textsuperscript{42}. HA-specific antibody was purchased from Covance (clone 1D6B12), and β-actin–specific antibody was purchased from Sigma (clone AC-74). HyT compounds were stored and aliquoted in DMDSO at 1,000× stock solutions.

Luciferase assay. Ten thousand stable HEK 293T cells infected with HA–luciferase–HaloTag were plated into each well of a 96-well plate. The next day, the HyT compounds indicated in Figure 1 were added in triplicate, and the cells were cultured for another 24 h. The cells were washed once with cold PBS and lysed in Passive Lysis Buffer (Promega). The luciferase activity was performed by Steady-Glo Luciferase Assay System (Promega) on a Wallac Victor 2 plate reader (Perkin Elmer), and the luciferase activity was normalized by protein concentration, as determined by the Bradford assay.

Immunoblotting. The indicated cells were washed twice with cold PBS, and the cells were lysed in lysis buffer (1× PBS, 1% NP-40, 10 mM EDTA, 40 mM HEPES) with protease inhibitors. The lysates were cleared by centrifugation at 10,000g for 5 min. The total protein concentration was determined by Bradford assay, and 50 μg of protein was loaded onto an 8% Bis-Tris gel. To solubilize polyubiquinated and aggregated proteins upon proteasome inhibition\textsuperscript{38}, samples were loaded with an SDS lysis buffer (1× PBS, 1% NP-40, 1% SDS, 1% sodium deoxycholate, 10 mM EDTA, 40 mM HEPES) with protease inhibitors (Fig. 2e). The blots were processed by standard procedures with the indicated antibodies (Fig. 2e), and the band intensities were quantified by ImageJ (US National Institutes of Health).

Flow cytometry analysis. Stable HeLa cells were raised by co-transfection of pcS2+ encoding EGFP–HaloTag and p-Puro, which contains the puromycin resistance gene. A chimeron population of cells expressing EGFP-HaloTag was isolated. These cells were treated with vehicle or 1 μM HyT13 for 24 h, washed with PBS and trypsinized. The cells were resuspended in FBS-free DMEM, and the intracellular EGFP level was measured by FACScalibur (BD Biosciences).

Zebranish. Danio rerio experiments. The wild-type fish line TLF was used for this study. The HA–HaloTag–Smad5 in PC2S+ was transcribed in vitro with the SP6 transcription kit (Ambion). The mRNA was injected at 10 ng μl\textsuperscript{−1} at the one-cell stage, and embryos were raised to the 256-cell stage, when they were moved to glass depression slides (ten per well) and put in 1 ml E2 medium (15 mM NaCl, 0.5 mM KCl, 1.0 mM MgSO\textsubscript{4}, 0.15 mM KH\textsubscript{2}PO\textsubscript{4}, 0.05 mM NaHPO\textsubscript{4}, 1.0 mM CaCl\textsubscript{2}, and 0.7 mM NaHCO\textsubscript{3}) with or without HyT13 (10 μM). Embryos were incubated at 28.6 °C for 24 h and then dechorionated and deyolked as previously described\textsuperscript{43}. Approximately 60 embryos per condition were collected for immunoblot analysis, as described above.

Focus formation assay. One hundred thousand NIH-3T3 cells infected with HA–HaloTag–Hras\textsuperscript{G12V} and HA–HaloTag\textsuperscript{G12V}-Hras\textsuperscript{G12V} were plated onto 10-cm cell culture plates in 10% PBS with DMEM. The next day, the medium was replaced with DMEM containing 1% FBS and the cells were administered either vehicle or 1 μM HyT13. The medium and the drug were replaced every 2 d. On day 6, the foci were photographed and counted as the number of distinct foci per 1 cm\textsuperscript{2}.

Tumor formation assay. One hundred thousand NIH-3T3 cells expressing HA–HaloTag–Hras\textsuperscript{G12V} were injected into the flank of anesthetized 6-week-old female nu/nu nude mice (Charles River Laboratories). Two hours later, the mice were i.p. injected with either vehicle (10 μl volume, 5 μl DMSO, 3 μl Cremophor EL (Sigma)), 25 mg per kg body weight HyT13 or 100 mg per kg body weight HyT13. The drug injections continued daily until the end of the experiment. Upon the appearance of tumors on day 7, the tumors were measured daily with calipers, and their volumes were calculated with the formula: ab\textsuperscript{3}/2, where ab represent the longest and shortest diameters of the tumor, respectively.

Animal experiments. All experimental protocols involving zebrafish and mouse work were performed under the auspices of Yale University’s Institutional Animal Care and Use Committee.

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Author contributions

T.K.N., H.S.T., A.R.S. and C.M.C. designed the research. T.K.N., H.S.T., A.R.S., M.J.S., T.W.C., T.B.S. and K.R. performed the experiments. T.K.N., H.S.T., A.R.S., M.J.S., and R. Weinberg at the Massachusetts Institute of Technology (MIT) contributed Addgene plasmid 9051 from which Hras1<sup>G12V</sup> was obtained. T.W.C., T.B.S. and K.R. performed the experiments. T.K.N., H.S.T., A.R.S. and C.M.C. analyzed the data. T.K.N., H.S.T. and C.M.C. wrote and edited the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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