Transformation by Hras[G12V] is Consistently Associated with Mutant Allele Copy Gains and is Reversed by Farnesyl Transferase Inhibition

Xu Chen[1], **Jacek M. Makarewicz**[1], **Jeffrey A. Knauf**[1,3], **Linda K. Johnson**[2], and **James A. Fagin**[1,3,4]

[1]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065

[2]Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY 10065

[3]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY 10065

[4]Weill-Cornell Medical College, New York, NY 10065

Abstract

RAS-driven malignancies remain a major therapeutic challenge. The two-stage 7,12-dimethylbenz(a)anthracene (DMBA)/12-o-tetradecanoylphorbol-13-acetate (TPA) model of mouse skin carcinogenesis has been used to study mechanisms of epithelial tumor development by oncogenic *Hras*. We used mice with a Hras[G12V] knock-in allele to elucidate the early events after Hras activation, and to evaluate the therapeutic effectiveness of farnesyltransferase (FTI) inhibition. Treatment of *Caggs-Cre/FR-Hras[G12V]* mice with TPA alone was sufficient to trigger papilloma development with shorter latency and a ~10-fold greater tumor burden than DMBA/TPA-treated WT controls. *Hras[G12V]* allele copy number was increased in all papillomas induced by TPA. DMBA/TPA treatment of *Hras[G12V]* knock-in mice induced an even greater incidence of papillomas, which either harbored *Hras[G12V]* amplification, or developed a *Hras[Q61L]* mutation in the second allele. Laser-capture microdissection of normal skin, hyperplastic skin and papillomas showed that amplification occurred only at the papilloma stage. *HRAS* mutant allelic imbalance was also observed in human cancer cell lines, consistent with a requirement for augmented oncogenic HRAS signaling for tumor development. The FTI SCH66336 blocks HRAS farnesylation and delocalizes it from the plasma membrane. NRAS and KRAS are not affected as they are alternatively prenylated. When tested in lines harboring *HRAS*, *NRAS* or *KRAS* mutations, SCH66336 delocalized, inhibited signaling and preferentially inhibited growth only of *HRAS*-mutant lines. Treatment with SCH66336 also induced near-complete regression of papillomas of TPA-treated *Hras[G12V]* knock-in mice. These data suggest that farnesyl transferase inhibitors should be reevaluated as targeted agents for human HRAS-driven cancers, such as those of bladder, thyroid and other epithelial lineages.

Correspondence: James A. Fagin, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 296, Zuckerman Bld, ZRC 504, New York, NY 10065, Phone: 646-888-2136, Faginj@mskcc.org.

Keywords

RAS; Papilloma; Farnesyl Transferase Inhibitor

Introduction

Hras, KrasA, KrasB and Nras are plasma membrane GTPases that exist in an active, GTP-bound, or inactive, GDP-bound, state. Many human tumors have a predilection for mutations in one *RAS* gene family member. *HRAS* mutations are less common overall, but they have a particularly high prevalence in cancers of the upper aerodigestive tract, skin, thyroid and urinary bladder. *KRAS* mutations predominate in pancreas, lung, and colorectal malignancies, whereas NRAS mutations are found in melanomas and hematopoietic tumors (1, 2).

Ras isoforms differ in their respective C-terminal hypervariable regions responsible for lipid modification, subcellular localization, intracellular processing and trafficking (3–7). It is clear that they have distinct functions, as knockout mice of each *Ras* gene display different phenotypes (8–10). However, there is no definitive explanation for the predilection for individual RAS oncogenes in different tumor lineages.

The two-stage model of mouse skin carcinogenesis has been extensively used for the study of tumor initiation, promotion and progression. In 7,12-dimethylbenz(a)anthracene (DMBA)-treated mouse skin, repeated topical applications of the tumor promoter 12-o-tetradecanoylphorbol-13-acetate (TPA) triggers skin papilloma development and progression into carcinomas. Mutant *Hras* alleles, mainly Q61L, are found in a high proportion of benign papillomas initiated by DMBA (11, 12). Targeted deletion of the *Hras* gene decreases the papilloma burden following a DMBA/TPA carcinogenesis protocol (13, 14), further establishing the importance of Hras in papilloma formation. These are often accompanied by an increase in copy number of the mutant allele (15). However, it is not clear whether *Hras* allelic imbalance is an obligate step in skin papilloma development. An increase of the mutant-to-wild-type *Hras* allelic ratio has been implicated in progression from squamous to spindle cell carcinomas (16). Loss of the wild-type *Hras* allele is also seen in papillomas after chemical skin carcinogenesis, leading to the presumption that the wild-type protein may function as a tumor suppressor. Recent data suggest that loss of the wild-type *Hras* allele may promote tumor progression rather than initiation (14). Despite the well-established role of *Hras* mutations in papilloma initiation in the skin 2-stage carcinogenesis model, Schuhmacher et al reported that papillomas failed to develop after topical administration of TPA to mice with an $Hras^{G12V}$ knock-in allele (17). In this paper we revisit this question in an independently derived $Hras^{G12V}$ knock-in mouse model of Costello's syndrome that spontaneously developed papillomas (18), and exploit this system to evaluate targeted therapeutics of Hras-driven tumors.

All Ras isoforms are farnesylated. Farnesyl transferase inhibitors (FTIs) block the addition of an isoprenoid group to the C-terminal portion of Ras to prevent formation of active Ras. FTIs block Hras farnesylation, membrane localization, and inhibit oncogenic Hras-driven cellular transformation *in vitro* (19, 20) and *in vivo* (21). However, in most clinical trials

FTIs showed no significant antitumor activity in patients with advanced solid tumors such as lung, pancreatic and colon cancers, which mainly harbor *KRAS* mutations (22–24), or with acute myeloid leukemia, which primarily have mutations of *NRAS* (25). The refractoriness to FTIs of RAS-driven cancers has been attributed to compensatory geranylgeranylprenylation of KRAS and NRAS, which preserves their membrane targeting and function (26–28). However, the HRAS selectivity of FTIs versus K- or NRAS-driven tumors has not been extensively studied in cells or in a mouse model, and no trial with an FTI has been done exclusively in patients with *HRAS* mutant tumors.

The FTI class of anti-cancer drugs has gone into disfavor in part because of the failure of clinical trials in patients that were not appropriately selected based on the oncogenic driver of the tumor. The data provided in this study present a strong preclinical rationale for revisiting the potential efficacy of FTIs in *HRAS* mutant tumors.

Results

Hras^{G12V} is sufficient to initiate skin tumorigenesis

We treated the skin of *Caggs-Cre/FR-Hras^{G12V}* mice, which have a knock-in *Hras^{G12V}* allele that is globally expressed at endogenous levels (18), with the tumor promoter TPA only and examined them for tumor development. As shown in Fig 1, topical administration of TPA alone to *Hras^{G12V}* mice is sufficient to trigger rapid papilloma development. The tumor latency is shorter and the incidence about 7-fold higher than in wild-type mice subjected to sequential DMBA/TPA treatment. However, combined DMBA/TPA treatment of *Hras^{G12V}* mice resulted in about 1.8-fold more tumors than those treated with TPA alone, indicating that DMBA cooperated with the endogenously activated mutant *Hras* allele in tumor initiation. Histological examination at week 15 showed TPA-induced hyperplastic changes in the epidermis and typical papillomas associated with increased Ki67 staining in *Hras*$^{G12V/}$ mice (Supplementary Fig 1). These observations indicate that oncogenic *Hras* is a tumor initiator and that it is sufficient to trigger the skin neoplastic process.

Hras allelic imbalance is an obligate step in papilloma development and occurs only at the papilloma stage

We previously reported that *Hras* gene copy number changes are almost always associated with the development of spontaneously-arising skin and forestomach papillomas, as well as angiosarcomas in *Hras^{G12V}* mice (18). As shown in Fig 2A, 10/10 papillomas arising in *Hras*^{G12V} mice treated with TPA alone had increased copies of the mutant *Hras* allele. By contrast, 7/10 randomly-selected papillomas induced by DMBA/TPA in *Hras^{G12V}* mice had increased copies of the *Hras^{G12V}* allele, whereas the remaining 3 had acquired an *Hras^{Q61L}* mutation (Fig 2B,C), which was located in the second *Hras* allele (Supplementary Fig 2). This helps to explain the greater tumor burden induced by DMBA/TPA in *Hras^{G12V}* mice compared to TPA alone (Fig 1). In DMBA/TPA-treated WT mice, 14/15 papillomas had only an *Hras^{Q61L}* mutation, as expected (See Table 1). To determine the stage of tumor development at which *Hras* mutant allelic imbalance takes place we performed a PCR-based analysis of laser-capture microdissected specimens of normal skin, hyperplastic skin and papillomas from TPA-treated *Hras^{G12V}* mice (Supplementary Fig 3). As shown in Fig 2D,

there was no gain in *Hras* mutant allele copy number in hyperplastic samples, whereas the adjacent papillomas from the same animals had a higher number of *Hras*^{G12V} alleles. Mutant *Hras* copy gain was also associated with increased expression of mutant *Hras* mRNA relative to wild-type (Supplementary Fig 4A and B). These data suggest that *Hras*-induced tumor formation requires augmented oncogenic Hras signaling, either through increased mutant *Hras* gene copy number or through acquisition of mutations in the second *Hras* allele. This also applies to the human cancer cell lines harboring *HRAS* mutations that we tested (Hth83, C643 and T24), all of which had two or more copies of mutant *HRAS* (Supplementary Fig 5).

The farnesyl transferase inhibitor SCH 66336 preferentially inhibits growth of HRAS-mutant human cancer cell lines

Covalent lipid modification of G proteins is required for their membrane localization. All RAS isoforms are farnesylated, whereas NRAS and KRAS, but not HRAS, are also weak substrates for geranylgeranylprenyltransferase-1 (GGTase-1) (26–28). The disappointing results of clinical trials with farnesyl transferase inhibitors (FTI) are thought to be due at least in part to the fact that they were tested in patients with *KRAS* or *NRAS* mutant cancers, in which the oncoprotein could still be prenylated and hence remain functional. We tested the effects of SCH 66336 on growth of 15 cell lines with different RAS mtations: 5 *HRAS*, 5°*NRAS* and 5 *KRAS*. As shown in Fig 3A, the 5 cancer cell lines with *HRAS* mutations were sensitive to the compound, with IC$_{50}$ ranging from 34 - 197nM. By contrast, the cell lines with *NRAS* or *KRAS* were mostly resistant, with 6 of them having an IC$_{50}$ > 600nM. These differences in FTI-responsiveness of *HRAS* vs *K-* or *NRAS* lines were also reflected in the growth curves (Fig 3B). Accordingly, SCH6636 also caused a profound inhibition of DNA synthesis in *HRAS*, but not in *KRAS* or *NRAS* mutant cells (Fig 3C and Fig 3D).

HRAS-specific delocalization and inhibition of MAPK signaling by SCH 66336

We next investigated whether the HRAS-selective effects on cell growth were also associated with corresponding effects on membrane targeting of the three RAS isoforms. As shown in Fig. 4A, SCH 66336 delocalized only HRAS from the membrane compartment to the cytosol of Hth83 cells, whereas KRAS and NRAS were unaffected. Treatment of Hth83 cells with SCH 66336 resulted in a time-dependent shift in migration of HRAS, which also accumulated over time (Fig 4B). This was already apparent 6h after addition of the compound, and peaked at about 48h. The timing of inhibition of pMEK closely paralleled HRAS defarnesylation, whereas cleavage of PARP was only apparent when pMEK was profoundly inhibited. We next performed dose-response studies of 4 *HRAS* and 4 *KRAS* or *NRAS* mutant lines incubated with SCH 66336 for 72h. SCH 66336 abrogated MAPK signaling, as determined by pMEK and pERK levels, in all 4 *HRAS* mutant lines in a dose-dependent manner, whereas AKT signaling was not decreased (Fig 4C). Interestingly, there was a paradoxical increase of pS473-AKT by the FTI in the *HRAS*-mutant Hth83 cells. This is likely an off-target effect, since knockdown of HRAS in these cells by RNA interference inhibited AKT phosphorylation at this site (Supplementary Figure 6B). SCH 66336 had no effect on MAPK signaling in cells with *KRAS* or *NRAS* mutation, despite inhibiting the farnesylation of wild-type HRAS (Fig 4D). Interestingly, although the *NRAS* mutant lines Hth7 and Act1 were relatively sensitive to the growth inhibitory effects of the compound

(IC$_{50}$ of ~ 200 nM; Fig 3), SCH 66336 did not inhibit pMEK in these lines even at the highest concentrations (1 μM). As Rheb GTPases are also farnesylated proteins, it is conceivable that the growth inhibitory effect of SCH 66336 on these *NRAS* mutant lines was exerted through their delocalization from endomembranes. This would be predicted to decrease mTOR activity, which was not observed, based on no detectable effect on phosphorylation of its substrate ribosomal S6. The mutant RAS dependency for signaling and growth is further demonstrated in Supplementary Fig 6 and Supplementary Fig 7. Thus, knock-down of mutant *HRAS* inhibited MAPK signaling and proliferation of Hth83 cells, whereas depletion of WT *KRAS* or *NRAS* had no appreciable effect on signaling and only a modest inhibitory effect on proliferation. Accordingly, in *KRAS*-mutant Cal62 cells and *NRAS* mutant Hth7 cells, only the respective mutant knockdown inhibited cell signaling and growth, whereas knockdown of the WT RAS isoforms was without effect on signaling and only modestly inhibited cell growth.

SCH 66336 induces regression of papillomas in Hras G12V mice

We next tested the effects of SCH 66336 on papillomas arising in 8 – 12 week-old *Hras^{G12V}* mice treated with TPA for 12 to 16 weeks. Mice were continuously treated with topical TPA, and randomized to vehicle or SCH66336 80mg/kg BID by gavage, and tumor growth monitored every 2–3 days by caliper measurements. As shown in Fig 5A and 5B, treatment of tumor-bearing mice with SCH 66336 induced an almost complete regression of the papillomas within 10 days. By contrast, tumors from mice in the vehicle-treated group continued to increase in size. Body weight was unchanged between both treatment groups. Immunohistochemical analysis showed that SCH66336-treated tumors had decreased Ki67 staining and lower pERK positivity (Fig 5C), consistent with the *in vitro* findings. Hepatocytes are the only cell type exhibiting clearly increased downstream signaling following endogenous expression of Hras^{G12V} *in vivo* (18). As shown in Fig 5D, SCH 66336 inhibited Hras farnesylation and decreased pERK in *Hras^{G12V}* mouse livers, consistent with appropriate targeting of the drug *in vivo*.

Discussion

Our studies show that endogenous expression of mutant Hras is likely sufficient to initiate skin tumorigenesis, as exposure of *Hras^{G12V}* mice to TPA alone resulted in papilloma development with shorter latency and greater tumor burden compared to DMBA/TPA treated *Hras-wt* mice. These results differ from those of a prior study in an independently-derived *Hras^{G12V}* knock-in mouse model, in which topical administration of TPA failed to induce papillomas (17). Although this could be due to a mouse strain effect, in our view it is more likely that the co-expressed IRES-β-geo cassette included in the 3′UTR of the Hras^{G12V} targeting vector in the Schuhmacher study may have interfered with endogenous expression of the oncoprotein, so that the required threshold for tumor initiation was not achieved. This is also supported by the fact that the *Hras^{G12V}-IRES-β-geo* mouse model had an attenuated developmental phenotype compared to the one described here.

Tumor initiation can be achieved by activating mutations or copy gains of oncogenes. We find that Hras-induced transformation is universally associated with copy gains of the

genomic locus containing the mutant allele, suggesting that *Hras* allelic imbalance is obligate for tumor development. The evidence for this is quite compelling. Thus, 10/10 papillomas arising in $Hras^{G12V}$ mice treated with TPA alone had increased copies of the mutant *Hras* allele. Moreover, all 10 randomly-selected papillomas induced by DMBA/TPA in $Hras^{G12V}$ mice had either increased copies of $Hras^{G12V}$ or an acquired *Q61L* mutation in the second *Hras* allele. All spontaneously arising skin papillomas, forestomach papillomas and angiosarcomas arising in these mice also had $Hras^{G12V}$ amplification (18). *Hras* mutant copy number increases have also been observed in DMBA/TPA-induced papillomas from wild-type mice (15). We also found *HRAS* copy gains in 2/3 human cancer cell lines we tested, with the other having a homozygous *HRAS* mutation, possibly due to uniparental disomy at that locus. *HRAS* activating mutations are also associated with gene copy gains in human Spitz nevi tumors (29) and human thyroid neoplasms (30). As might be predicted, *Hras* mutant allelic imbalance was not present in normal skin or in hyperplastic specimens, whereas it was consistently found in papilloma tissues. The association of oncogene mutation and amplification is not uncommon. For instance, an increase in *EGFR* (31) or *KRAS* (32) mutant allele copy number is seen in many non-small cell lung cancers. However, mutant allelic imbalance is not required for transformation, as many NSCLCs harboring these mutations have a single copy of the oncogene.

The role of wild-type *Ras* alleles in cell transformation is complex (33). On the one hand, wild type *Kras* and *Nras* can act as tumor suppressors in cells with a corresponding mutant *Ras* allele (34), an effect that has been shown to be dose-dependent (35, 36). Our results suggest that loss of the wild-type *Hras* allele is not required for transformation of the skin epithelium by mutant *Hras*, as only a minor subset of papillomas had loss of WT *Hras* in $Hras^{G12V}$ knock in mice (18). Recent data from the Balmain lab is consistent with these findings, as *Hras* heterozygosity appears to be primarily involved in malignant transformation of skin tumors, rather than on tumor initiation (14). We found that *WT-HRAS* alleles are present in most human cancer cell lines harboring *HRAS* mutations. Instead, our results suggest that intensification of mutant Hras signaling through copy-number imbalances may play a key, and possibly rate-limiting, role in papilloma initiation, as supported by the fact that this imbalance is only seen at the papilloma stage. This was also noted in Rat 1 fibroblasts harboring a single $Hras^{G12V}$ mutant allele introduced by homologous recombination (37). Finney and Bishop found that endogenous $Hras^{G12V}$ expression was not sufficient to induce Rat 1 fibroblast transformation *in vitro*, whereas cells commonly had excess copies of the mutant *Hras* allele when transformed clones developed after serial passaging.

We found that knockdown of Hras or Kras in cell lines expressing the corresponding mutant oncoprotein markedly decreased basal MAPK signaling, whereas individual knockdown of the wild type Ras isoforms did not. These data do not challenge the conclusions of recent studies that point to an important supportive role of wild-type Ras in signaling activated by oncogenic Ras (38–40), since we did not attempt to deplete all wild-type Ras isoforms. Silencing of oncogenic Ras in three different *RAS*-mutant cell lines inhibited cell growth more profoundly than depletion of each of the wild-type Ras isoforms, although the latter

also dampened cell growth to a modest extent, consistent with a cooperative role of wild-type Ras proteins in tumorigenesis (38–40).

All Ras isoforms (Hras, Kras and Nras) are farnesylated. Although ~30% of human cancers harbor *RAS* mutations, the therapeutic efficacy of FTIs in the treatment of cancer patients has been disappointing (22–25). We found that human cancer cell lines harboring *HRAS* mutations are more sensitive to the antiproliferative effects of the FTI SCH 66336 than those carrying *KRAS* or *NRAS* mutations. Using FTI SCH 66336 across a panel of 5 different cell lines from different tissues with *HRAS* mutation, the FTI reduced growth at IC50s ranging from 34 – 197nM. By contrast, the cell lines with *NRAS* or *KRAS* were mostly resistant, with six of them having an $IC_{50} > 600$nM (Fig. 3). Treatment of Hras^{G12V} mice harboring papillomas with SCH66336 resulted in profound tumor regression associated with inhibition of Hras downstream signaling. Although this effect is likely exerted through inhibition of Hras signaling in the transformed epithelial cells, we cannot exclude an effect of the FTI on stromal cells or on the tumor vasculature, as the mutant allele is ubiquitously expressed.

The failure of clinical trials with FTIs can now be explained based on a better understanding of their mechanism of action. Unlike HRAS, which is exclusively farnesylated, KRAS and NRAS can be prenylated by geranylgeranyl transferases upon blockade of farnesylation, and remain functional (27, 28). Consistent with this, we found that SCH 66336 delocalized HRAS, but not KRAS or NRAS, from the membrane to the cytoplasm, and abrogated its downstream signaling via MAPK. Clinical trials with FTIs did not consider this mutational specificity in their criteria for selecting patients for enrolment. This was in part due to preclinical studies that showed that some cell lines with *KRAS* or *NRAS* mutations, or even cells that were wild-type for all *RAS* oncogenes, were variably growth inhibited by FTIs. Bernhard et al showed that an FTI inhibitor, when used at concentrations that inhibited farnesylation but not geranylgeranylation, increased the radiosensitivity of two *HRAS* mutant lines but not two *KRAS* mutant cell lines (41). By contrast, Sepp-Lorenzino et al noted that the effects of a peptidomimetic FTI were unrelated to the *RAS* mutation status of the cells. In hindsight, this is not surprising as there were no *HRAS* mutant cell lines used in their experiments (42). The FTI SCH 66336 was found to selectively delocalize *HRAS* and induce G1/S arrest in cell lines expressing oncogenic *HRAS*, while showing inhibitory effects on the G2-M transition and on anchorage-independent growth in cells with *NRAS* mutation or that were wild type for *RAS* (43). These and other preclinical reports, and the fact that *KRAS* and *NRAS* are mutated far more frequently in human cancers than *HRAS*, may have led to these agents being tested in trials with patients selected irrespective of tumor genotype. Hence, the clinical activity of these compounds has not yet been evaluated in patients harboring the cancers that are most likely to respond.

A recent clinical trial with the FTI lonafarnib shows that benefits can be derived when the patient population is selected based on a better understanding of the therapeutic target. Children with the Hutchinson-Gilford progeria syndrome harbor a germline mutation in *LMNA* which disables a proteolytic cleavage site in lamin A required to remove its farnesylated C-terminus during its post-translational processing. Treatment with the FTI lonafarnib showed improvement of vascular stiffness, bone structure and audiological status in a significant fraction of these children (44). According to the COSMIC database, the

frequency of *HRAS* mutations is about 15 % in salivary tumors, 10% in tumors of the urinary tract, 8% in tumors of the cervix and upper aerodigestive tract and about 4% in prostate and thyroid cancers. In certain subtypes of thyroid cancer the prevalence of *HRAS* mutation is particularly high, such as in sporadic medullary thyroid cancers (45, 46). Based on all these considerations, we propose that FTIs should be reappraised in properly designed clinical trials against their validated therapeutic targets.

Materials and Methods

Experimental Animals, Skin Carcinogenesis and Drug administration

FR-*Hras* ^{G12V} mice, which harbor a latent *Hras*^{G12V} knock-in allele under the regulatory control of its endogenous gene promoter, were crossed with *Caggs-Cre* mice to obtain widespread endogenous expression levels of oncogenic Hras (18). Six to eight weeks old mice were shaved before a single 25 μg dose of DMBA (Sigma-Aldrich) was applied to the dorsal skin. A week later, TPA (12.5 μg, Sigma-Aldrich) was applied twice per week for 15 weeks. The farnesyl transferase inhibitor SCH 66336 (Merck) was dissolved in 20% (wt/vol) 2-hydroxylpropyl-β-cyclodextrin and administered to mice b.i.d by oral gavage at a dose of 80mg/kg. Skin tumors were monitored and recorded and measured twice per week for the duration of the studies.

Tissue preparation, histopathology and immunohistochemistry

Mice were killed by CO_2 asphyxiation. Normal and tumor tissue lysates were prepared for extraction of RNA, DNA or protein as described (18). Histology was performed on H&E-stained formalin-fixed paraffin embedded sections. Antibodies used for IHC are listed in the SI section. Animal care and all procedures were approved by the MSKCC Institutional Animal Care and Use Committee.

Hras Allelic Imbalance Analysis

The genomic DNA from tissues was used as a template for PCR amplification with primers that distinguish mutant (666 bp product due to insertion of *loxp*) from WT *Hras* alleles (622 bp). The primers and PCR conditions were previously described (18).

Laser-Capture Microdissection

Mouse skin was promptly excised after killing and immediately frozen with liquid N_2. Frozen sections (8 μm were used to microdissect skin epithelial cells of normal, hyperplastic and papilloma tissues by laser capture using an Arcturus microdissection system. DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN).

Cell culture

The following cancer cell lines were used in this study: *HRAS* mutant: Hth83 and C643 (thyroid), Hs578T (breast), SK-Mel-31 (melanoma), T24 (bladder); *KRAS* mutant: Cal62 (thyroid), H441, A549 and H2030 (lung), Hct116 (colorectal); *NRAS* mutant: ACT1 and Hth7 (thyroid), Sk-Mel-30, Sk-Mel-130 and SK-Mel-173 (melanoma). Culture conditions are described in the SI materials and methods. For proliferation assays, cells were plated in

triplicate into 6-well plates at 5×10^4 cells/well, and treated the next day with or without SCH 66336 for the indicated times, with media changes every day. Cells were collected by trypsinization and counted in a Vi-Cell cell viability analyzer (Beckman Coulter). IC_{50} values were calculated using Prism software. For Western blotting, cell extracts and immunoblotting were performed as described (18). For antibody details, see Supplementary Information.

Bromodeoxyuridine (BrdU) labeling

Cells were seeded in 6-well plates with 1.5×10^5 cells per well 1 day before treatment with SCH 66336 for 72 h. Cells were then incubated with 40 μM BrdU for 4 h. Cell fixation and labeling with anti-BrdU fluorescein isothiocyanate (FITC) conjugated antibody and 7-aminoactinomycin D (7-AAD) were performed using the FITC BrdU Flow Kit (BD Pharmingen) according to the manufacturer's instructions. The percentage of BrdU positive cells was determined by fluorescence –activated cell sorting analysis (FACS) using a Cell Lab Quanta flow cytometer (Beckman Coulter).

Cell Fractionation

Subcellular fractionation was carried out through sequential incubation with cytosolic, membrane and nuclear fraction extraction buffers, using the Subcellular Protein Fractionation Kit of Thermo Scientific. Fractions were subjected to Western blotting with the indicated antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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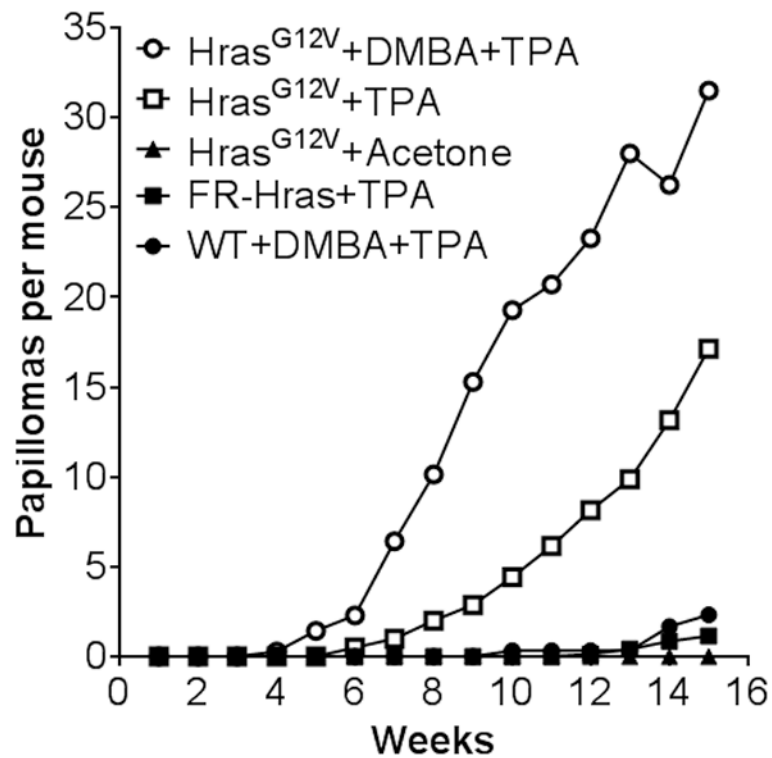
Fig. 1. Topical administration of TPA to Hras^{G12V} mice triggers papilloma development
Average number of tumors per mouse during the time course of the study. There were five groups of mice: Hras^{G12V} mice treated with a single dose of DMBA followed by TPA treatment twice per week (n=7), Hras ^{G12V} mice treated with TPA only (n=7), wild type mice treated DMBA followed by TPA (n=7), Hras ^{G12V} mice treated with the vehicle acetone (twice per week) (n=8) and *FR-Hras* mice (harboring the targeted *Hras* allele, unrecombined, because of the absence of *Caggs-Cre*) treated with TPA (n=7).
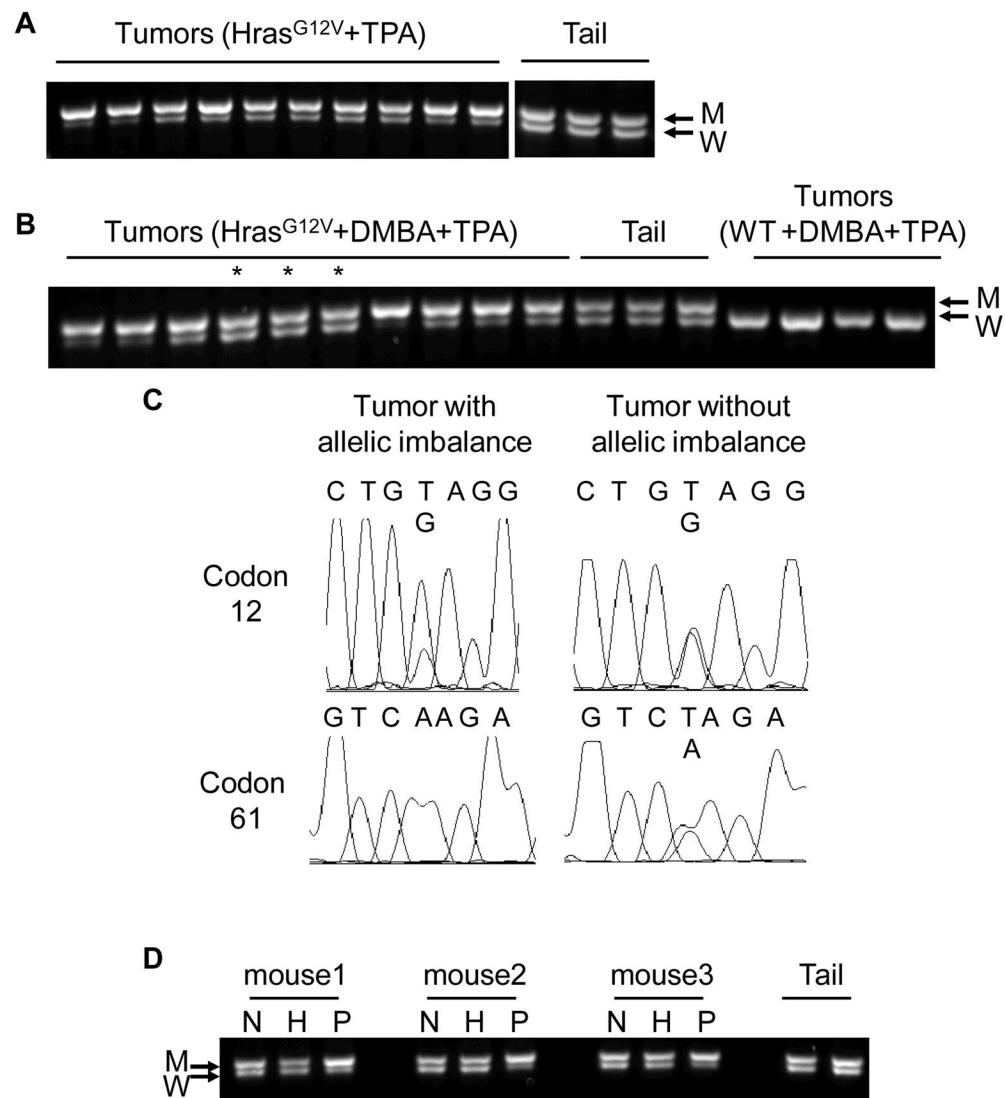
Fig. 2. *Hras* allelic imbalance in papillomas from *Hras*^G12V mice

A) PCR of genomic DNA of 10 papillomas or tails from TPA-treated Hras^{G12V} mice with primers that distinguish mutant from WT *Hras* alleles. W: wild-type 622 bp allele; M: 666bp targeted allele. The mutant *Hras* allele copy number was increased in all 10 papillomas induced by TPA alone. B) PCR of DNA from 10 randomly selected papillomas from DMBA/TPA-treated Hras^{G12V} mice, tails from Hras^{G12V} mice and papillomas from DMBA/TPA treated WT mice. 7/10 papillomas had M>W allelic imbalance. The three lanes marked by an asterisk had ~ 1/1 ratio of M/W alleles. C) Representative *Hras* Sanger sequence traces of PCR products of tumor DNAs from panel B: *Left*: Tumor with M>W *Hras* allelic imbalance, showing the *Hras G12V* mutation, and a WT *Hras Q61* codon. *Right*: Tumor (*) without *Hras* allelic imbalance (M=W), harboring both *Hras G12V* and *Q61*L mutations. *G12V* and *Q61L* are located in different alleles (Suppl Fig 2). D) Laser-capture microdissection (LCM) of normal skin epithelium, hyperplasia and papilloma. PCR of genomic DNA of LCM of tissues from TPA-treated Hras^{G12V} mice. As indicated, *Hras*

mutant allele amplification occurred only at the papilloma stage. N: normal skin epithelium; H: hyperplasia; P: papilloma.
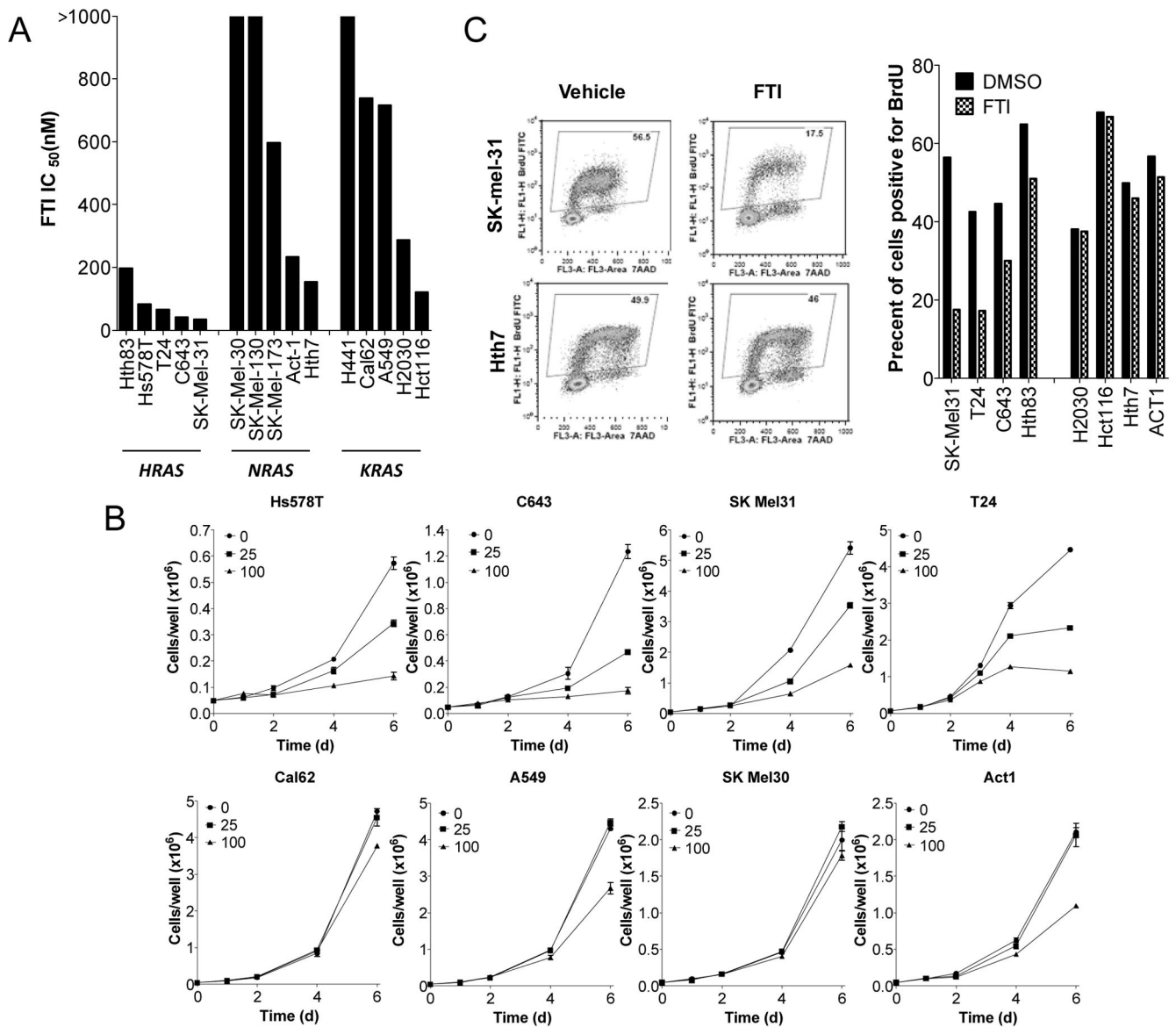
Fig. 3. Growth of human cancer cell lines with *HRAS* mutation is preferentially inhibited by SCH 66336

A) IC_{50} of each cell line for SCH 66336. The indicated cell lines were incubated with different concentrations of SCH 66336 for 4 days and the cell number counted. All 5 *HRAS* mutant cell lines have lower IC_{50} for FTI compared to cells with *NRAS* or *KRAS* mutation.
B) Growth of cell lines in the presence of SCH 66336. The indicated cell lines were incubated with SCH 66336 (25nM, 100nM) or vehicle for 2, 4 and 6 days. C) *Left panel*: FACS for FITC-labeled BrdU vs 7-AAD for representative *HRAS* (SKmel31) and *NRAS* (Hth7) mutant cell lines treated for 3 days with 100 nM SCH 66336. *Right panel*: SCH 66336 decreased DNA synthesis in the 4 *HRAS* mutant but not in *NRAS* or *KRAS* mutant cell lines.
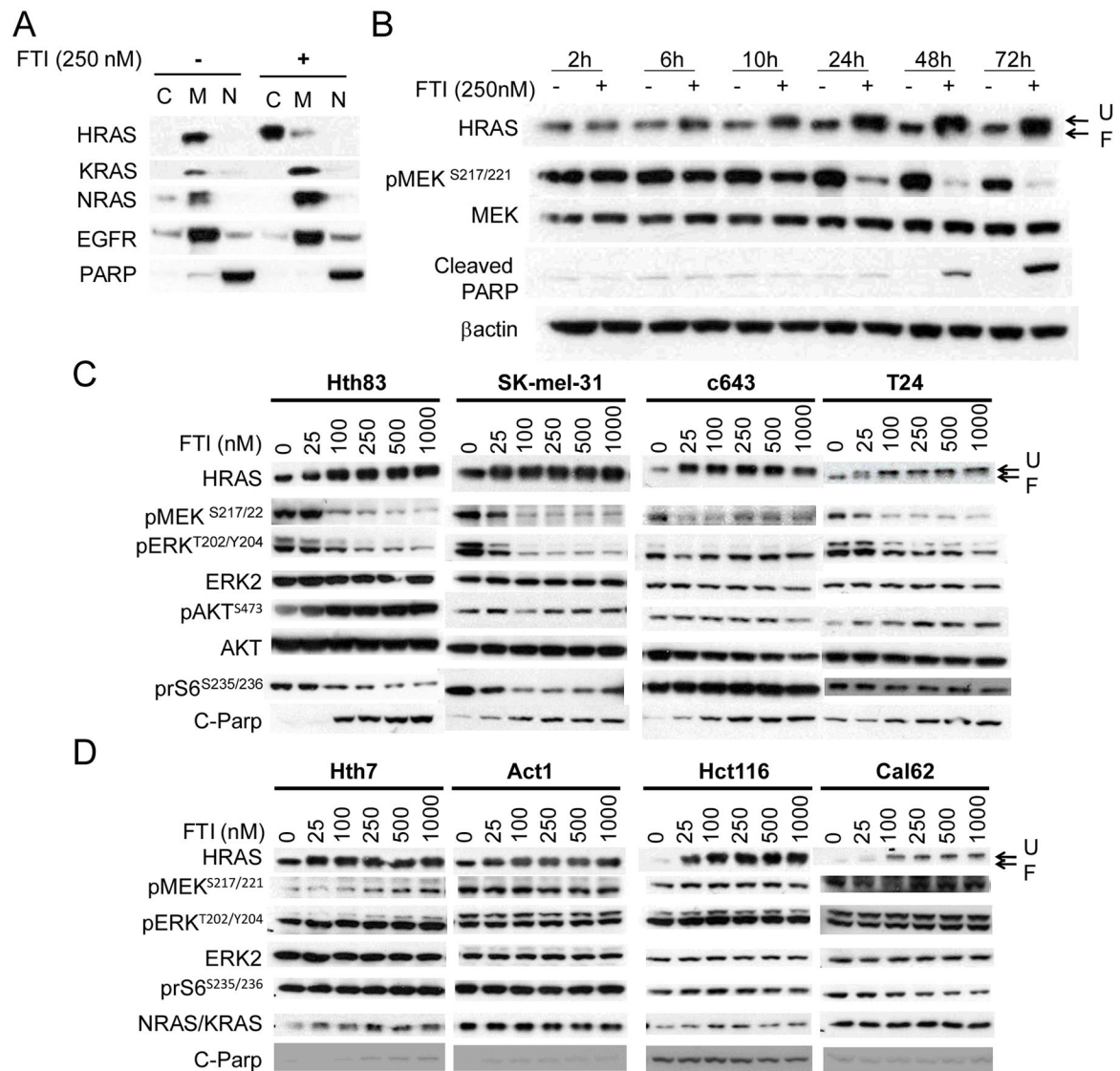
Fig. 4. SCH 66336 disrupts HRAS membrane localization and inhibits MAPK signaling in *HRAS* mutant lines

A) Western blots of subcellular fractions of Hth83 cells treated with 250nM SCH 66336 for 48hr, probed with the indicated antibodies. SCH 66336 delocalized HRAS, but not KRAS or NRAS. C: Cytoplasmic; M: Membrane; N: Nucleus. B) SCH 66336 inhibits HRAS signaling with a delayed time course in Hth83 cells. Hth83 cells were incubated with 250nM SCH 66336 for the indicated time. The cell lysates were Western blotted with the indicated antibodies. U: unfarnesylated HRAS; F: farnesylated HRAS. C) SCH 66336 blocked MAPK signaling in a concentration-dependent manner. Western blots of 4 HRAS mutant cell lines treated with the indicated concentration of SCH 66336 for 72hr probed with indicated antibodies. D) SCH 66336 had no effect on MAPK signaling in cell lines with KRAS or NRAS mutations, despite inhibiting farnesylation of WT HRAS in these lines.
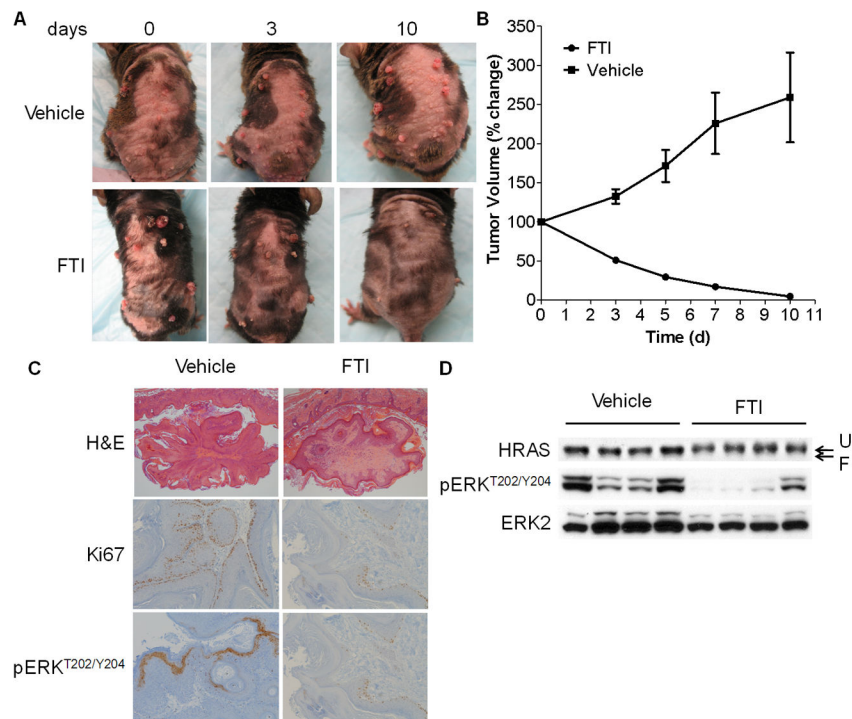
Fig. 5. SCH 66336 induces regression of papillomas in *Hras^{G12V}* mice

A) Representative photos of mice after treatment with vehicle or SCH 66336 for the indicated times. *Hras^{G12V}* mice were first treated with TPA for 12 to 16 weeks to induce papilloma development. Mice then were treated with 80mg/kg SCH 66336 b.i.d. (n=5) or vehicle (n=6) by gavage for the indicated times. B) Tumor volumes at the indicated times during treatment with vehicle (n=10) or SCH 66336 (n=11) from the mice described above. Tumor size was measured on days 0, 3, 5, 7 and 10. Bars represent mean ± SEM. C) SCH 66336 decreased cell proliferation and pERK in papillomas. Representative H&E and IHC staining for Ki67 and pERK in sections of mouse papillomas treated with vehicle or SCH 66336. D) Western blot of liver tissues from Hras^{G12V} mice treated with vehicle (n=4) or SCH 66336 (n=4), probed with the indicated antibodies. SCH 66336 decreased MAPK signaling and inhibited Hras farnesylation in livers of Hras^{G12V} mice.

Table 1

Hras

Gene in Papillomas of $Hras^{G12V/+}$ Mice Treated With DMBA/TPA

	$Hras^{G12V}$ mutation	$Hras^{G12V}$ copy number increase	$Hras^{Q61L}$ mutation
$Hras^{G12V}$+DMBA+TPA	10/10	7/10	3/10
$Hras^{G12V}$+TPA	10/10	10/10	0/10
$Hras^{WT}$+DMBA+TPA	0/15	nd	14/15