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IQGAP1 scaffold-kinase interaction blockade selectively targets RAS-MAP kinase–driven tumors

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Abstract

Upregulation of the ERK1 and ERK2 (ERK1/2) MAP kinase (MAPK) cascade occurs in >30% of cancers¹, often through mutational activation of receptor tyrosine kinases or other upstream genes, including *KRAS* and *BRAF*². Efforts to target endogenous MAPKs are challenged by the fact that these kinases are required for viability in mammals^{3,4}. Additionally, the effectiveness of new inhibitors of mutant BRAF has been diminished by acquired tumor resistance through selection for BRAF-independent mechanisms of ERK1/2 induction^{2,5,6}. Furthermore, recently identified ERK1/2-inducing mutations in MEK1 and MEK2 (MEK1/2) MAPK genes in melanoma confer resistance to emerging therapeutic MEK inhibitors, underscoring the challenges facing direct kinase inhibition in cancer^{7,8}. MAPK scaffolds, such as IQ motif-containing GTPase activating protein 1 (IQGAP1)^{9,10}, assemble pathway kinases to affect signal transmission^{11–13}, and disrupting scaffold function therefore offers an orthogonal approach to MAPK cascade inhibition. Consistent with this, we found a requirement for IQGAP1 in RAS-driven tumorigenesis in mouse and human tissue. In addition, the ERK1/2-binding¹⁴ IQGAP1 WW domain peptide disrupted

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AUTHOR CONTRIBUTIONS

K.L.J. conceived the study and developed crucial proof-of-concept studies.

K.L.J. also performed and designed all IQGAP1 knockout *in vivo* characterization, IQGAP1 RNAi tissue and cell studies, designed and constructed the WW lentivirus, performed WW peptide validation in cells, tissue and *in vivo* mouse models and did immunoprecipitation studies. K.L.J. contributed to PLX-4032-resistant experiments, clinical chemistry data analysis and pancreatic cancer studies. Additionally, K.L.J. coordinated all aspects of the project, interpreted data and wrote the manuscript. P.K.M. performed and designed the pancreatic cancer studies and edited the manuscript. A.M.Z. performed and designed PLX-4032-resistant experiments, contributed to cell studies, pancreatic cancer studies, bioinformatics analysis and clinical chemistry data analysis and edited the manuscript. J.Z. performed bioinformatics analysis and edited the manuscript. B.Z. performed IQGAP1 rescue experiments and contributed to immunoprecipitation studies. J.S. supervised the pancreatic cancer studies, interpreted data and helped write the manuscript. P.A.K. supervised the project, interpreted data and wrote the manuscript.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

IQGAP1-ERK1/2 interactions, inhibited RAS- and RAF-driven tumorigenesis, bypassed acquired resistance to the BRAF inhibitor vemurafenib (PLX-4032) and acted as a systemically deliverable therapeutic to significantly increase the lifespan of tumor-bearing mice. Scaffold-kinase interaction blockade acts by a mechanism distinct from direct kinase inhibition and may be a strategy to target overactive oncogenic kinase cascades in cancer.

Intracellular scaffold proteins interact with kinases and other signaling molecules to control signal transduction^{11–13}. IQGAP1 is a scaffold that is known to interact with RAF, MEK1/2 and ERK1/2 MAPK cascade kinases, as well as with a host of other proteins^{9,10,15}. Some studies have suggested a role for IQGAP1 in enhancing tumorigenesis^{16–19}, but *Iqgap1* knockout mice are viable and fertile²⁰, do not show any defects in normal epithelium and heal wounds normally (**Supplementary Fig. 1**). Thus, IQGAP1 is a potential tumor-required scaffold protein that is dispensable for homeostasis.

To further explore this possibility functionally, we examined the susceptibility of *Iqgap1*-null mice to tumor formation in a model of RAS-driven cancer. We observed that *Iqgap1* knockout mice were resistant to Hras-driven chemical carcinogenesis²¹ (**Fig. 1a,b** and **Supplementary Fig. 2**). *Iqgap1* heterozygotes showed an intermediate phenotype, suggesting an *Iqgap1* gene-dosage effect on tumor susceptibility (**Fig. 1**). *Iqgap1* knockout mice also had a sixfold reduction in premalignant epidermal hyperplasia in response to acute Ras-MAPK induction²² (**Supplementary Fig. 3**). Expression profiling of the skin of these mice demonstrated that *Iqgap1* is required for Ras-driven induction of genes enriched in cellular metabolism Gene Ontology terms (**Supplementary Fig. 4**) that are characteristic of early Ras-MAPK-driven tumorigenesis²³. *Iqgap1* knockout mice did not differ substantially from wild-type mice in the expression of known *Iqgap1* binding partners, suggesting that *Iqgap1* is not required for their expression (**Supplementary Table 1**).

We then assessed *Iqgap1* expression in spontaneous human epidermal cancers to examine the corresponding human skin cancer. *Iqgap1* protein was strongly expressed in 45% of human squamous cell carcinomas ($n = 60$) (**Supplementary Fig. 5**), consistent with reports in other cancers^{24,25}. In a mouse model²⁶ of human *KRAS*-driven pancreatic ductal adenocarcinoma²⁷, we found that the amount of *Iqgap1* protein is increased in pre-neoplasms and advanced tumors (**Supplementary Fig. 6**). To further explore the effects of *Iqgap1* in human tissue, we depleted *Iqgap1* in RAS-driven organotypic human epidermal neoplasia²⁸, a setting that reflects corresponding human cancers, and found that *Iqgap1* depletion impaired basement membrane degradation and neoplastic invasion (**Fig. 2a**). Similarly to in mice, IQGAP1-deficient human epidermis was normal, in contrast to the severe hypoplasia seen in ERK1/2-deficient tissue (**Supplementary Fig. 7**). Although hypoplasia induced by loss of ERK1/2 has been shown to be associated with reduced cyclin B1 and c-Fos expression leading to G2/M arrest⁴, their expression remains unchanged with *Iqgap1* loss. Total ERK1/2 depletion led to hypoplastic tissue collapse in RAS-driven organotypic neoplasia, whereas depletion of *Iqgap1* preserved tissue morphology (**Fig. 2b**). Although both total and activated phosphorylated ERK1/2 (pERK1/2) were absent in ERK1/2-depleted tissue, IQGAP1-deficient tissue retained some pERK1/2, indicating that *Iqgap1* loss down-regulates pERK1/2 but does not eliminate it (**Fig. 2b**), an effect that is

rescued by *Iqgap1* re-expression (**Supplementary Fig. 8**). Further, *Iqgap1* depletion diminished downstream ERK1/2 cascade activity (**Supplementary Fig. 9**). *Iqgap1* depletion thus lowers the amounts of active ERK1/2 and impedes neoplasia without inducing tissue catastrophe.

The effects of *Iqgap1* on pERK1/2 suggested that the anti-neoplastic effects of *Iqgap1* loss may occur through alterations in ERK1/2. *Iqgap1* binds ERK1/2 through its highly conserved WW domain (**Fig. 2c**). Although other *Iqgap1* domains are known to bind multiple partners in other signaling pathways¹⁵, ERK1/2 are the only known proteins that have been confirmed to bind the IQGAP1 WW sequence¹⁴. Moreover, WW-deleted *Iqgap1* mutants do not rescue IQGAP1-mediated effects on pERK1/2²⁹. Similarly to scaffold loss, increased scaffold concentrations can also alter signaling pathways by titrating binding partners away from one another^{12,13}, and both overexpression and knockdown of IQGAP1 show similar effects on signal transduction²⁹. We therefore used the ERK1/2-binding WW sequence as an approach to target IQGAP1-ERK1/2 interactions. Similarly to IQGAP1 depletion, lentiviral expression of a Myc-tagged WW sequence inhibited neoplastic invasion in organotypic neoplasia (**Fig. 2d,e** and **Supplementary Figs. 8 and 9**). Substitution of two conserved WW-domain tyrosines to alanine (**Fig. 2c**) abolished this effect (**Fig. 2d,e** and **Supplementary Fig. 8**).

To characterize the action of the IQGAP1 WW domain in additional cancer cell types, we delivered it by lentivirus to cancer cell lines with mutational RAS-MAPK pathway activation (MDA-MB-468, HCT-116 and COLO-829), as well as to cancer cell lines with a wild-type MAPK module (T-47D, COLO-320DM and CHL-1) (**Supplementary Fig. 10a,b**). The WW domain markedly impaired the proliferation of breast, colorectal and melanoma tumor cells characterized by *EGFR* overexpression, *KRAS* mutation and *BRAF* mutation, respectively, without affecting wild-type MAPK lines (**Supplementary Fig. 10c,d**). Moreover, the WW domain impaired migration of the MAPK mutant lines with no effect on wild-type lines (**Supplementary Fig. 10e-h**). We did not find any changes in IQGAP1, IQGAP2 or IQGAP3 expression levels in these cells (**Supplementary Fig. 11**). To further explore selectivity, we used melanoma cancer cell lines previously characterized to express either the *BRAF*^{V600E} or *NRAS*^{Q61L} mutant (COLO-829 and MM-485, respectively), as well as a control melanoma line with wild-type *BRAF* and *NRAS* (CHL-1). IQGAP1 WW domain delivery impaired tumorigenesis in the *BRAF*- and *NRAS*-mutant cells but not in RAS-MAPK wild-type tumor cells (**Supplementary Fig. 12**), again suggesting a selective inhibitory effect on neoplasias characterized by mutations upstream of ERK1/2 in the RAS-MAPK pathway. We then performed intratumoral lentiviral injection to determine whether the WW domain can act on established tumors. Subcutaneous tumors derived from human MAPK-mutant breast and melanoma skin cancer cell lines (MDA-MB-468 and COLO-829, respectively) treated with WW lentivirus ceased to grow, whereas control lentivector-injected tumors continued to expand (**Supplementary Fig. 13**). The IQGAP1 WW sequence thus exerts anti-neoplastic effects on not only MAPK pathway-mutant early stage tumors but also established tumors.

To test the antitumor activity of the IQGAP1 WW peptide itself, we rendered the 32-residue WW peptide cell and tissue penetrating by adding eight arginine residues in an approach that

mimics HIV TAT sequence function³⁰. WW peptide, but not scrambled peptide control, abolished neoplastic invasion in RAS-driven organotypic neoplasia (**Fig. 3a**). The WW peptide reduced the amounts of pERK1/2 in oncogenic RAS-transformed keratinocytes and MAPK-mutant cancer cell lines (**Supplementary Fig. 14**) and impaired the growth of human pancreatic cancer cell lines characterized by mutant *KRAS* (**Supplementary Fig. 15**). Further, systemic WW peptide delivery by osmotic pump impaired tumorigenesis in mice bearing SK-Mel-28 melanoma tumors, as well as those bearing MDA-MB-468 breast tumors (**Supplementary Fig. 16**). We did not find any morbidity or substantial laboratory abnormalities in WW peptide-treated mice (**Supplementary Table 2**).

We next treated *Ptfla*^{+/-Cre}; *Kras*^{+/-G12D}; *Trp53*^{flox/flox} mutant mice, which develop lethal pancreatic cancer²⁶, with the WW peptide starting at 35 days of age, when palpable tumor mass was present. Compared to treatment with scrambled peptide or gemcitabine, which is commonly used in human pancreatic cancer, WW treatment significantly extended lifespan (**Fig. 3b,c** and **Supplementary Table 3**). Visual and histopathological analysis indicated that although WW-treated mice eventually die from pancreatic tumors, these tumors show reduced amounts of pERK1/2, suggesting that the WW peptide sustains inactivation of the Ras-MAPK pathway (**Fig. 3d,e** and **Supplementary Fig. 17**). Thus, systemically delivered Iqgap1 WW peptide shows marked antitumor activity without detectable toxicity.

The newly available kinase inhibitor vemurafenib (PLX-4032) targets mutant BRAF^{V600E} MAP3K in melanoma; however, its long-term effectiveness has been hindered by acquired resistance through bypass mechanisms that can restore ERK1/2 activation^{2,5,6,8}. If the WW domain acts in a mechanistically distinct fashion from direct kinase inhibition, then the effects of the WW peptide should be unaltered by acquired resistance to BRAF inhibition. We therefore compared the impact of the WW peptide on PLX-4032-sensitive and -resistant melanoma lines. Three PLX-4032-sensitive BRAF^{V600E}-expressing parental human melanoma cancer cell lines, which are all sensitive to the WW peptide (**Fig. 4a,b**; COLO-829, SK-Mel-28 and A375), were rendered resistant to PLX-4032 as described^{5,6}. Resistance of the resulting subclones to PLX-4032-mediated growth inhibition (**Fig. 4c,d**) was accompanied by alterations that have been previously associated with the emergence of PLX-4032 resistance^{5,6}, including alterations of insulin-like growth factor 1 receptor (IGF1-R), platelet-derived growth factor receptor-β (PDGFR-β), NRAS and COT (also called MAP3K8) (**Supplementary Fig. 18**). In all cases, the WW peptide inhibited the growth of PLX-4032-resistant melanoma subclones (**Fig. 4c**) similarly to in PLX-4032-sensitive parental cells (**Fig. 4a**), indicating that the WW domain acts in a non-redundant fashion to direct kinase inhibition.

To further explore the mechanism of WW peptide action, we next characterized its effects on IQGAP1-ERK binding in primary melanocytes and BRAF^{V600E}-expressing human melanoma cancer cells. The WW peptide substantially decreased the amounts of total ERK1/2 bound to IQGAP1 in cancer cells (**Fig. 4e,f**). Notably, very little ERK1/2 was associated with IQGAP1 in primary cells, whereas there was much more ERK1/2 bound to IQGAP1 in cancer cells (**Fig. 4e,f** and **Supplementary Fig. 19**). The *Iqgap1* WW sequence thus blocks scaffold-kinase interactions between IQGAP1 and ERK1/2.

Here we show that the IQGAP1 scaffold is required for tumorigenesis but is dispensable for homeostasis and the IQGAP1 WW sequence blocks neoplasia without inducing the tissue collapse accompanying ERK1/2 ablation. We also present a working model of the resulting scaffold-kinase interaction blockade (SKIB) in cancer (**Fig. 4g**). The WW peptide inhibited tumorigenesis in cells from diverse tissues, all of which were characterized by RAS-MAPK pathway activation at a variety of pathway levels, ranging from upstream receptor tyrosine kinases to commonly mutated *RAS* and *RAF* genes. Exogenous WW peptide bypassed acquired resistance to vemurafenib (PLX-4032)^{5,6}, a recently approved treatment of BRAF^{V600E} melanoma, suggesting that this approach may be explored in tumors with acquired resistance to RAS-MAPK pathway-targeted drugs. Systemic delivery of a tissue-penetrating Iqgap1 WW peptide impaired growth of established tumors and increased the lifespan of tumor-bearing mice without detectable toxicity. These data support future efforts at therapeutic targeting of scaffold-kinase interactions as a tumor-selective strategy to inhibit oncogenic signaling in cancer.

ONLINE METHODS

Mouse handling and genotyping

All mouse husbandry and experimental procedures were performed in accordance and compliance with policies approved by the Stanford University Administrative Panel on Laboratory Animal Care (Khavari lab protocol #9863 or Sage lab protocol #13565). Mice were housed and bred under standard conditions with food and water provided *ad libitum* and maintained on a 12-h light, 12-h dark cycle. *Iqgap1* knockout mice were maintained in a C57BL/6 and 129 mixed background²⁰. Genotyping was performed using genomic DNA isolated from mouse tails in Direct PCR lysis reagent (Viagen). The primers used for gene amplification were p5, 5'-TTGCAGTCTGTGGCATGTG-3' and p3, 5'-CCTGCTGACAGGTCAATGAT-3' for the wild-type *Iqgap1* allele or p5, 5'-TTGCAGTCTGTGGCATGTG-3' and pNeo, 5'-CCTGCTCTTTACTGAAGGCT-3' for the neomycin cassette, as previously described²⁰. K14-ER:RAS transgenic mice, Jax stock number 006403, were kept in a 129/SvEv background²². This line was crossed to *Iqgap1* knockout mice and subsequently backcrossed to C57BL/6 and 129 wild-type mice. The ER:RAS transgene was detected with the following primers: forward, 5'-CACCACCAGCTCCACTTCAGCACATT-3' and reverse, 5'-CGCACCAACGTGTAGAAGGCATCCTC-3'. *KRAS*^{+LSL-G12D} (ref. 31), *Ptf1a*^{+Cre} (ref. 32) and *p53*^{fllox/fllox} (ref. 33) mice were maintained as previously described.

Wound healing

Two round, full-thickness excision wounds 6 mm in diameter were made on the dorsal skin of anesthetized 6- to 8-week-old mice using sterile biopsy punches. Untreated wounds were measured for closure and photographed at least once every 3 d. Percentage wound closure was calculated as the wound area remaining over the initial wound size.

7,12-dimethylbenz(a)anthracene (DMBA) and TPA chemical carcinogenesis

Skin carcinogenesis was as previously described³⁴. Briefly, the backs of anesthetized 8- to 10-week-old mice were shaved and treated two times (3 d apart) with application of DMBA

(Sigma; 10 µg in 100 µl acetone) followed by twice weekly application of TPA (Sigma; 12.5 µg in 100 µl acetone). Tumors were observed twice per week for 30 weeks and recorded by photography and caliper measurements of length, width and height. Tumor volume was estimated using the formula $\frac{4}{3}\pi(r^1r^2r^3)$, where r^1 is the radius of length, r^2 is the radius of width and r^3 is the radius of height. Gender-matched littermates were used for all experiments.

Acute oncogenic RAS

Exposure to acute oncogenic RAS was as previously described²². Briefly, the lower backs of anesthetized 6- to 8-week-old mice were shaved and treated for 6 d with once-daily application of 1 mg 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) in 0.1 ml ethanol to activate the ER:RAS transgene. Relative hyperproliferation was quantified by the thickness of the interfollicular epidermis from the basal layer to the top of the cornified layer using a micrometer and measured ten times across three independent fields of view. Values were normalized to control untreated wild-type mouse skin. Gender-matched littermates were used for all experiments.

Gene expression profiling

Fragmented complementary RNA was hybridized to the Mouse Gene 1.0 ST Array (Affymetrix). For microarray gene expression analysis, arrays were robust multiarray average (RMA) normalized, and differential expression was defined using a fold change of at least four after 6 d of topical treatment with 4-OHT compared to untreated samples. A total of 402 genes were identified as the most significantly differentially expressed between K14-ER:HRAS^{G12V} *Iqgap1*^{+/+} and *Iqgap1*^{-/-} mice. Pathway analysis was then performed using DAVID. Gene expression data are deposited in the Gene Expression Omnibus under accession code GSE44967.

Isolation and culture of primary human cells and human cancer cell lines

Primary human epidermal keratinocytes and melanocytes as well as dermal fibroblasts were isolated from discarded neonatal surgical specimens and cultured as previously described²⁸. Human cancer cell lines were maintained in DMEM or RPMI (Gibco) supplemented with 10% FBS or FCS according to recommendations from ATCC.

Human tissue model system

Human tissue was regenerated as previously described²⁸. Briefly, stromal primary human fibroblasts were seeded onto devitalized human dermis and subsequently elevated to a sterilized annular dermal support (ADS) tissue culture insert device²⁸. Primary keratinocytes were transduced with viral constructs as previously described²⁸. These cells were then seeded to the air-liquid interface of the upper chamber of the ADS insert, with the medium changed daily. In response to 4-OHT (Sigma; 100 nM in ethanol), cells expressing inducible ER-HRAS^{G12V} invaded into the underlying dermis within ~5 d. Immunofluorescence was performed as previously described³. Microscopy was performed on an Olympus FV1000 scanning laser confocal microscope. For each sample, 30 sequential Z sections were taken in three channels (DAPI: 405 nm/450 nm (excitation/emission); FITC: 488 nm/ 512 nm; and

TRITC: 540 nm/570 nm), allowing for subsequent three-dimensional reconstruction analysis. Image analysis and three-dimensional reconstruction were performed using Improvision Volocity software (PerkinElmer). The x-y planes are shown in three-color images (red, green and blue), and the z-x planes are restricted to two colors (red and green) for ease of viewing effects on the basement membrane. The relative invasion index was quantified as the number of keratin-positive epithelial cells below the collagen-positive basement membrane measured ten times across three independent fields of view.

IQGAP1 knockdown vectors

The siRNA oligonucleotides targeting IQGAP1 were designed and synthesized by Dharmacon: siIQGAP1.a, 5'-GAACGUGGCUUAUGAGUACUU-3' and 5'-GUACUCAUAAGCCACGUUCUU-3' and siIQGAP1.b, 5'-CCUCUCGCUCUGAUGGGACAUUUGU-3' and 5'-ACAAAUGUCCCAUCAGAGCGAGAGG-3' targeting the 3' untranslated region of endogenous IQGAP1. ERK1/2 and nontargeting control siRNAs were synthesized as previously described⁴. One million early passage neonatal keratinocytes were electroporated with 2 nM total siRNA using Amaxa nucleofection reagents according to the manufacturer's protocol. The vectors for pGIPZ shRNA targeting IQGAP1 were designed and purchased through Open Biosystems, cat nos. V2LHS-86779 (shIQGAP1.a), V2LHS-86781 (shIQGAP1.b) and V2LHS-259635 (shIQGAP1.c).

Western blots

Cells were lysed after treatment as indicated in NP-40 buffer plus phosphatase and protease inhibitors (Roche). Immunoblots were performed as previously described³ with the following antibodies: rabbit antibody to ERK1/2 (1:1,000, Cell Signaling, 9102), rabbit antibody to pERK1/2 (Thr202/Tyr204; 1:1,000, Cell Signaling, 9101), rabbit antibody to ERK2 (1:1,000, Santa Cruz Biotechnology, 153), mouse antibody to IQGAP1 (1:400, Upstate, 05-504), rabbit antibody to IQGAP1 (1:500, Abcam, 86064), rabbit antibody to Myc (1:500, Abcam, 9106), rabbit antibody to PDGFR- β (1:1,000, Cell Signaling, 3169), rabbit antibody to IGF1-R (1:1,000, Cell Signaling, 3018) and mouse antibody to hemagglutinin (HA) (1:2,000, Covance, MMS-101P).

Quantitative RT-PCR (qRT-PCR)

At the indicated time points, RNA was harvested from cells by treatment with TRIzol according to the standard Invitrogen protocol. Relative mRNA expression was determined by qRT-PCR analysis using a Stratagene Mx3000P thermocycler and Brilliant II SYBR Green qRT-PCR one-step master mix reagents. The primer concentration was 200 nM with 100 ng RNA. Samples were run in triplicate and normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S mRNA for each reaction. The following primers were used for this study: IQGAP1: forward, 5'-TTCCGCACTACCCAGACCTTGTTT-3' and reverse, 5'-CCTGTCTTGGATGTGGCCTTTGG-3'; IQGAP2: forward, 5'-TCTGTGCCACTTAGAGGAAGCCAA-3' and reverse, 5'-CACTGGACGGTATTATCTGTGTGTCGAA-3'; IQGAP3: forward, 5'-AGGGCAAGGCAGCCCAGACT-3' and reverse, 5'-GCCGTTGGCACAGTCGGGAA-3'; Myc- tagged

WW: forward, 5'-GATAATAACAGCAAGTGGGTG-3' and reverse, 5'-GGGTCTCCAGATTGTGGTAATAA-3'; Myc-tagged mutant WW: forward, 5'-TTCTGAAGAAGATCTGGATA-3' and reverse, 5'-CCAGATTGTGGGCAG CATAA-3'; and COT: forward, 5'-CAAGTGAAGAGCCAGCAGTTT-3' and reverse, 5'-GCAAGCAAATCCTCCACAGTTC-3' (previously described⁶)

Rescue

Primary human keratinocytes were infected with pLEX-ER: HRAS^{GV12}, and after puromycin selection, cells were treated with either empty virus control or pLEX-HA-IQGAP1. After site-directed mutagenesis to remove an internal BamHI site in the host pCR-BluntII-TOPO *Iqgap1* (Open Biosystems, cat. no. MHS4426-99626202) vector, full-length IQGAP1 was PCR amplified with 5' BamHI and 3' XhoI primers and cloned in frame into pLEX-N-terminal-HA-tagged vector. Seventy-two hours after infection, cells were nucleofected with siRNA targeting a control sequence or IQGAP1.b. Seventy-two hours after nucleofection, cells were harvested for western blot analysis. Samples were immunoblotted as described above.

Immunoprecipitation-kinase assay

Cell Signaling p44/42 MAP Kinase Assay Kit immunoprecipitation-kinase assays were used to evaluate perturbations to ERK1/2 MAPK biochemical signaling after ablation of ERK MAPK scaffolds according to the manufacturer's protocol. Briefly, genetically modified cells were lysed under non-denaturing conditions at defined time points after treatment. Cell lysates were incubated overnight at 4 °C in the presence of immobilized monoclonal primary antibody to pERK1/2. Immunoprecipitated complexes were then mixed with an ELK-1 fusion protein and cold ATP. Samples were analyzed by gel electrophoresis and immunoblotting for mouse pELK-1 (Ser383; 1:1,000, Cell Signaling, 9186).

Immunohistochemistry

Immunohistochemistry was performed as previously described³ with the following antibodies: rabbit antibody to pERK (Thr202/Tyr204; 1:25, Cell Signaling, 4370), rabbit antibody to ERK (1:100, Cell Signaling, 4695), mouse antibody to IQGAP1 (1:25, Invitrogen, 33-8900), rabbit antibody to Myc (1:100, Abcam, 9106) and rabbit antibody to cleaved caspase 3 (1:400, Cell Signaling, 9664). The skin cancer and normal tissue microarrays SK208 and SK2081 (Biomax) were scored blindly on the basis of IQGAP1 stain.

Coimmunoprecipitation

Cells were crosslinked with 1 ml of 20 mM dithiobis[succinimidyl propionate] (DSP) (Thermo Scientific) for 1 h at 4 °C, and the reaction was quenched with washes in 50 mM Tris. Cells were lysed in 20 mM Tris, 150 mM NaCl, 0.2% NP-40 and 10% glycerol, with 0.5 mM dTT and protease and phosphatase inhibitors (Roche) added directly before use (buffer 1) for 1 h at 4 °C but not pelleted. One milligram lysate in 500 µl was combined with 5 µg of mouse antibody to IQGAP1 (Millipore, 05-504) in buffer 1 and rocked overnight at 4 °C. Thirty microliters of protein G sepharose 4 fast flow (GE Healthcare) was washed in

buffer 1 and combined with lysate for 1 h at 4 °C. Supernatant was removed to check for immunodepletion. Beads were washed three times in 20 mM Tris, 150 mM NaCl and 1% Triton X-100, with protease and phosphatase inhibitors added directly before use (buffer 2). Immunoprecipitate was eluted from beads in 4× LDS sample buffer in buffer 1 plus 5% Mercaptoethanol. Samples were immunoblotted as described above.

Cloning, mutagenesis and peptide production of the IQGAP1 WW domain

The pLEX-Myc WW clone was PCR amplified from pCR-BluntII-TOPO IQGAP1 (Open Biosystems, cat. no. MHS4426-99626202) using the following primers: forward, 5'-GCTCGCGGATCCACCATGGAACAAAACTTATTTCTGAAGAAGATCTGGATAATAACAGCAAGTGGGTGAAGCAC-3' and reverse, 5'-ATAAGTGC GGCCGCTTATGGGGGTTTCATCCCATCCTCCTTCCTG-3'. PCR products were subsequently digested with BamHI and NotI restriction enzymes and ligated into the LentiORF pLEX-MCS vector (Open Biosystems, cat. no. OHS4735). To generate the pLEX mutant WW clone, QuikChange site-directed mutagenesis (Stratagene) was used. The following primers were used to mutate Tyr696 and Tyr697 to alanine: forward, 5'-GGTGAAGCACTGGGTAAAAGGTGGATATTATGCTGCCCAATCTGGAGACC-3' and reverse, 5'-GGTCTCCAGATTGTGGGCAGCATAATATCCACCTTTTACCCAGTGCTTCACC-3'. Peptides corresponding to octo-arginine Myc-tagged scrambled control ((D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)) EQKLISEEDLSHGNQWDWKYLGNYKVTGNEVYEDKGYEPHWP), to octo-arginine Myc-tagged WW domain ((D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)) EQKLISEEDLDNNSKWVKHWVKG GY Y Y Y H N L E T Q E G G W D E P P)) and to octo-arginine Myc-tagged mutant WW domain ((D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)(D-arginine)) EQKLISEEDLDNNSKWVKHWVKG GY Y A A H N L E T Q E G G W D E P P)) were synthesized by Biomatik, CSBio and Stanford Biomaterials and Advanced Drug Discovery laboratories in acetate salt and resuspended in water to a 2.5–5 mM working solution. Peptide was added directly into the medium of tissue culture or into osmotic pumps at the concentrations indicated.

Cell viability assay

Genetically modified adherent cancer cell lines were seeded in equivalent, low-density cultures in duplicate in 24-well plates. Twenty-four hours after seeding, the medium was removed and replaced with 500 µl of a 5:1 mixture of cell medium and cell titer blue reagent (Promega). Cultures were incubated with this mixture for 2 h at 37 °C in the dark. Fluorescence (560 nm/590 nm (emission/excitation)) for 100 µl in triplicates for each sample was recorded. Incubation with cell titer blue reagent and fluorescent readings were repeated every 2 d for a total of 12 d. Values plotted correspond to readings taken while control samples were still growing exponentially. For pancreatic cancer cell lines (Panc1, CFPac1 and AsPc1), WW or SCR peptide was added at a concentration of 20 µM. The MTT cell proliferation assay (Roche) was performed in triplicate according to the manufacturer's instructions. For PLX-4032-resistant subclones (adapted from ref. 6), cultured cells were

seeded into 96-well plates (250–3,000 cells per well). Twenty-four hours after seeding, serial dilutions of PLX-4032 were prepared in DMSO and added to cells, yielding final drug concentrations ranging from 0 to 50 μM PLX-4032, with the final volume of DMSO not exceeding 1%. Cells were treated daily and incubated for 72 h after the addition of drug. Data from growth-inhibition assays were modeled using a nonlinear regression curve fit with a sigmoid dose response. These curves were displayed, and half-maximal inhibitory concentration (IC_{50}) values were generated using Prism 5 (GraphPad). Sigmoid-response curves that crossed the 50% inhibition point at or above 20 μM have IC_{50} values designated as $>20 \mu\text{M}$.

Migration assay

Ibidi silicone culture inserts were used to evaluate migration without variable scratching and physical wounds to cells and used according to the manufacturer's protocol. Briefly, cells were seeded at 25,000–50,000 cells per chamber and allowed to attach overnight. Twenty-four hours later, the culture inserts were removed, and the cells were treated with mitomycin C to inhibit proliferation. Cells were cultured under normal conditions, and images were acquired at the time points indicated until control samples had fully migrated into the opening. Light microscopy images were acquired (three per well and three replicates per sample). Images were analyzed using automated image analysis software (Ibidi, Wimasis), and migration was quantified relative to empty vector control.

Xenografts, *in vivo* treatments and measurements

One-million transduced cancer cells engineered were suspended in a volume of 200 μl containing 50% Matrigel (BD Biosciences) and injected with a 27-G needle into the subcutaneous space of hairless 6- to 8-week-old severe combined immuno-deficient (SCID) mice (SHO stock, Charles Rivers). For melanoma tumor growth experiments, cells were transduced with luciferase and empty vector or WW before injection. For intratumoral injection experiments, cells were transduced with luciferase, and xenografts were allowed to grow untreated for 3 weeks. Subsequently, tumors were measured and equally divided depending on size into two groups. Each group received five treatments of 200 μl lentiviral empty vector or WW intratumorally injected every 3 d over the course of 2 weeks. For osmotic pump experiments, cells were transduced with luciferase, and xenografts were allowed to grow untreated for 1–2 weeks. Subsequently, tumors were measured and equally divided depending on size into two to three groups. Osmotic pumps (Alzet) loaded with 20 mg ml^{-1} peptide were implanted into the subcutaneous space of the mice. In all conditions, tumors were observed at least twice per week and recorded by photography and caliper measurements of length, width and depth. Tumor volume was estimated using the formula $\frac{4}{3}\pi(r^1r^2r^3)$, where r^1 is the radius of length, r^2 is the radius of width and r^3 is the radius of height. Bioluminescence imaging was performed using an IVIS-200 imaging system (Xenogen Corporation). After anesthesia with 2.5% isofluorane, mice were injected intraperitoneally with 150 mg per kg body weight D-luciferin in PBS. Plateau phase-emitted light was captured with an open emission filter as the average radiance (photons per second per cm^2 per steradian). For pancreatic cancer *in vivo* models, mice were treated with either the WW peptide (50 mg per kg body weight twice daily), a control scrambled peptide (50 mg per kg body weight twice daily) or 100 mg per kg body weight of gemcitabine (Sigma)

dissolved in saline with scrambled peptide by intraperitoneal injection. Gemcitabine was used on a Q3D×4 schedule (every third day for four cycles) as previously described³⁵.

Chemical and hematological parameters

Whole blood was obtained by cardiac puncture under isoflurane anesthesia before euthanasia and submitted for routine hematologic and biochemical analysis.

Derivation of PLX-4032-resistant tumors

Cells were made resistant to PLX-4032 as previously described^{5,6}. Briefly, BRAF^{V600E} cancer cell lines were grown in low doses of PLX-4032 (1 μ M in DMSO; Active Biochem) for 6–8 weeks until the amounts of pERK were increased and cells were no longer sensitive to increased doses of PLX-4032 (5 μ M). From these resistant stocks, cells were plated in limiting dilution in 96-well plates to isolate single cell colonies. These colonies were then assayed for resistance to PLX-4032, as well as recognized mechanisms of resistance, and used for subsequent proliferation assays after treatment with peptide as indicated.

DNA sequencing reactions

RNA was isolated from PLX-4032-sensitive parental and resistant subclones using TRIzol and converted to complementary DNA (cDNA). The cDNA was used as a template to amplify *NRAS* using *NRAS* forward (5'-CTGAGTACAACTGGTGGTGGTT-3') and reverse (5'-GGCTTGT TTTGTATCAACTGTCC-3') primers, as well as the following protocol: a single step at 94 °C for 3 min and then 35 cycles of amplification (94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min). PCR products were gel purified and submitted for Sanger sequencing (5'-GTACAACTGGTGGTGGTTGG-3').

Statistics

Each data panel is representative of at least three independent replicate experiments or multiple independent mice, as indicated. The s.d. was used for all analyses. Further analysis of variance and subsequent *post hoc* comparisons used Student's *t* tests. Kaplan-Meier survival data were reanalyzed using a log-rank test.

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Figure 1.

Diminished tumorigenesis in *Iqgap1* knockout mice. **(a)** Representative clinical appearance of tumors in mice wild type (left), heterozygous (middle) or null (right) for *Iqgap1* over time as indicated (two mice in each group are shown at each time point). Scale bar, 5 mm. **(b)** Quantification of the formation of the tumors in **a** over time. Color bars denote tumor volume, as indicated in the legend; abortive papillomas <1 mm³ are not shown ($n = 7$ mice per genotype). TPA, 12-O-tetradecanoylphorbol-13-acetate.

Figure 2.

The ERK-binding WW domain of IQGAP1 inhibits RAS-driven invasion in human tissue.

(a) Confocal stack images showing the expression of keratin (red), basement membrane protein type VII collagen (Col VII; green) and the nuclei of keratinocytes and fibroblasts (blue) of RAS-driven organotypic human epidermal neoplasia treated with shRNAs targeting scrambled control (shControl) or IQGAP1 (shIQGAP1.a, shIQGAP1.b and shIQGAP1.c). The images are shown on the *x-y* plane (top) or the *z-x* plane (middle); note the blockade of invasive destruction of the basement membrane by tissue depletion of IQGAP1. Scale bars (top and middle), 25 μ m. On the bottom is immunohistochemistry staining for IQGAP1. The black dashed lines denote the basement membrane separation of the epidermis and underlying dermis. Scale bar (bottom), 25 μ m. (b) Immunohistochemistry staining for pERK1/2 (top) and total ERK1/2 (bottom) of the tissue in a treated with siRNAs targeting a nonfunctional control (siControl), ERK1/2 (siERK1/2) or IQGAP1 (siIQGAP1.a) sequence. Black arrowheads denote residual pERK staining in siIQGAP1.a-treated tissue. The black dashed lines denote the basement membrane. Scale bar, 25 μ m. (c) Cross-species conservation of the IQGAP1 WW domain; divergent amino acids are listed in red. Pink shading indicates highly conserved residues across the WW domains from multiple proteins. Red asterisks denote residues mutated in the mutant WW domain construct. aa, amino acids. (d) Confocal stacks colored as in a after treatment with lentivirus corresponding to empty vector control, the IQGAP1 WW domain (WW) or the WW domain mutant with mutation of the two tyrosine residues noted in c (mutant WW). Scale bars, 25 μ m. (e) Immunoblots for

the Myc tag, pERK1/2 and total ERK1/2 in the cells in **d**. Cell lysates were also probed with antibodies to actin to verify equal loading.

Figure 3.

Exogenous WW peptide delivery inhibits neoplastic invasion and diminishes tumorigenesis *in vivo*. **(a)** Expression of keratin (red), basement membrane protein type VII collagen (green) and the nuclei of keratinocytes and fibroblasts (blue) of RAS-driven organotypic human epidermal neoplasia treated with 10 μM Myc-tagged scrambled control (SCR peptide) or 10 μM Myc-tagged IQGAP1 WW (WW peptide) daily. Images are shown in the *x-y* plane (first row) or the *z-x* plane (second row). Also shown is immunohistochemistry staining for the Myc tag (third row), pERK1/2 (fourth row) and total ERK1/2 (fifth row). Black arrowheads denote residual pERK staining in WW peptide tissue. All scale bars, 25 μm. **(b)** Survival of 35-day-old *Ptf1a*^{+/Cre}; *Kras*^{+/G12D}; *Trp53*^{fl/fl} mice with established tumor burden and palpable pancreas mass randomized to receive WW peptide (WW), scrambled peptide placebo (SCR) or gemcitabine by intraperitoneal injection. **(c)** Median survival of the mice in **b**. Statistical significance was calculated by log-rank test. NS, not significant. Data are shown as the mean ± s.d. **(d)** Clinical images of tumors from the mice in **b**. Scale bars, 1 cm. Black arrowheads denote tumor nodules. s, spleen; d, duodenum. The WW peptide-treated tumor is from day 99, and the SCR-treated control tumor is from day 51. **(e)** Immunohistochemistry staining for the Myc tag of tissue from the mice in **b** along with an untreated control mouse.

Figure 4.

Exogenous WW peptide bypasses PLX-4032 resistance. **(a)** Viability of PLX-4032-sensitive BRAF^{V600E} melanoma parental cell lines treated with DMSO, 5 μM PLX-4032, 10 μM scrambled peptide or 10 μM WW peptide, as indicated. **(b)** Dose response of the cells in **a** to increasing doses of PLX-4032 (0–50 μM). **(c)** Viability of PLX-4032-resistant subclones derived from the parental lines in **a** treated with DMSO, 5 μM PLX-4032, 10 μM scrambled peptide or 10 μM WW peptide, as indicated. **(d)** Dose response of the cells in **c** to increasing doses of PLX-4032 (0–50 μM). Data **(a–d)** are shown as the mean ± s.d. **(e)** Immunoprecipitation (IP) of IQGAP1 protein and associated ERK1/2 from human primary melanocytes (MC) or BRAF^{V600E} SK-Mel-28 melanoma cells after daily treatment with 10 μM scramble control (SCR) or WW peptide for 6 d. **(f)** Quantification of the relative amounts of ERK1/2 as a function of the immunoprecipitated IQGAP1 in **e**. **(g)** Model of WW-mediated SKIB action in RAS-MAPK-hyperactive cancer. In untreated cancer cells (left), *Iqgap1* scaffolds RAF, MEK and ERK MAPKs and facilitates neoplasia-enabling signal transduction. In the presence of WW SKIB (right), ERK MAPK is sequestered away from the IQGAP1 scaffold, the amounts of pERK1/2 are diminished and neoplastic response is attenuated.