Inhibition of RAS-Mediated Transformation and Tumorigenesis by Targeting the Downstream E3 Ubiquitin Ligase Seven in Absentia Homologue

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Abstract

Constitutively active RAS small GTPases promote the genesis of human cancers. An important goal in cancer biology is to identify means of countervailing activated RAS signaling to reverse malignant transformation. Oncogenic K-RAS mutations are found in virtually all pancreatic adenocarcinomas, making the RAS pathway an ideal target for therapeutic intervention. How to best contravene hyperactivated RAS signaling has remained elusive in human pancreatic cancers. Guided by the Drosophila studies, we reasoned that a downstream mediator of RAS signals might be a suitable anti-RAS target. The E3 ubiquitin ligase seven in absentia (SINA) is an essential downstream component of the Drosophila RAS signal transduction pathway. Thus, we determined the roles of the conserved human homologues of SINA, SIAHs, in mammalian RAS signaling and RAS-mediated tumorigenesis. We report that similar to its Drosophila counterpart, human SIAH is also required for oncogenic RAS signaling in pancreatic cancer. Inhibiting SIAH-dependent proteolysis blocked RAS-mediated focus formation in fibroblasts and abolished the tumor growth of human pancreatic cancer cells in soft agar as well as in athymic nude mice. Given the high level of conservation of RAS and SIAH function, our study provides useful insights into altered proteolysis in the RAS pathway in tumor initiation, progression, and oncogenesis. By targeting SIAH, we have found a novel means to contravene oncogenic RAS signaling and block RAS-mediated transformation/tumorigenesis. Thus, SIAH may offer a novel therapeutic target to halt tumor growth and ameliorate RAS-mediated pancreatic cancer.

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Introduction

RAS proteins are evolutionarily conserved small GTPases that shuttle between a GTP-bound active state and GDP-bound inactive state (1, 2). The RAS signaling pathway controls cell proliferation, differentiation, and survival in all multicellular organisms (3–6). Consequently, proper regulation of RAS signaling is critically important for normal development, and dysregulation of the RAS pathway leads to pathologic diseases (7–9). Indeed, constitutively active RAS promotes cell proliferation, neoplastic transformation, and tumorigenesis (7, 10, 11). Oncogenic RAS proteins are commonly detected in human cancers including ~90% of pancreatic cancers, ~70% of malignant neoplasias and ~30% of all human cancers (10, 12–14). Despite the central importance of RAS signaling in human cancer, direct inhibition of hyperactivated RAS has not achieved sufficient clinical efficacy (8, 11, 15). We reasoned that targeting a highly conserved downstream signaling module of the RAS pathway might provide a more effective strategy for inhibiting oncogenic RAS signaling and RAS-mediated tumorigenesis in human cancer.

Signaling events downstream of RAS are complex, nonlinear, and dynamic (16, 17). RAS transmits signals from receptor tyrosine kinases to the nucleus through multiple downstream signaling pathways, chiefly the RAF/MEK/ERK, PI3K/AKT, and RalGEF pathways (4, 6, 18–20). In addition, genome-wide surveys have found numerous putative RAS target genes mediating RAS transformation (21, 22). Despite the identification of these diverse RAS targets, it is still difficult to precisely determine which downstream signaling components represent the best therapeutic targets for blocking RAS-mediated neoplastic transformation/tumorigenesis. Thus, identifying the ideal downstream targets in RAS signal transduction to best contravene oncogenic RAS signaling remains a major challenge in cancer biology.

To address this problem, we exploited the wealth of valuable information and insights gained from genetically tractable model organisms. Genetic studies of Drosophila photoreceptor development have identified many downstream components of the RAS pathway as well as described how the major signaling modules are assembled together (23). The Drosophila studies reveal that the proper transmission of RAS/RAF/MEK/ERK signals requires an E3 ligase, seven in absentia (SINA) being the most downstream component identified thus far (23, 24). Indeed, loss-of-function sina mutations block the ability of activated RAS/RAF/MEK/ERK to specify the R7 neuronal cell fate by preventing the RAS-regulated proteolysis of SINA substrates (23, 25). Thus, the SINA-dependent proteolytic pathway seems to serve as a necessary downstream gatekeeper required for proper RAS signal transduction in Drosophila (25).

SINA belongs to an evolutionarily conserved family of E3 ubiquitin ligases. The human genome has two seven in absentia homologues (SIAH1/2) which share >83% amino acid identity with the Drosophila SINA (ref. 26; Supplementary Fig. S1A). Whether human SIAH is a bona fide signaling component of the mammalian RAS pathway is unclear. Due to the extraordinary amino acid conservation shared among the SINA/SIAH E3 ligases, it is conceivable that SIAH-dependent proteolysis might be similarly required for mammalian RAS signal transduction as described in flies. SINA/SIAHs have been implicated in numerous cellular...
Figure 1. SIAH is expressed in proliferating cells. A. SIAH expression in human cancer cells. H&E. H&E staining for the serial paraffin sections of an invasive grade 3 (of 4) human colorectal adenocarcinoma. H&E staining was used to view tissue morphology/cell histology. The section reflects a part of the tumor mass that was defined as a villous adenoma located in the cecum. SIAH, SIAH staining. A majority of the tumor cells in the colorectal adenoma stain with SIAH. The normal differentiated colorectal epithelium cells show little or no background SIAH staining whereas the basal undifferentiated and proliferating columnar epithelium cells show some SIAH staining, indicating that the antibodies are specific for proliferating cells and do not stain normal and nondividing cells. Ki67, as a control, a majority of SIAH (+) tumor cells were also stained positive for Ki67, a well-established marker for proliferating cells. B. Representative image showing predominantly nuclear SIAH localization in human pancreatic cancer cells (CAPAN-2) as visualized by (b1) SIAH, (b2) Ki67, (b3) DAPI, and (b4) merge. C. Western blot analysis indicates that endogenous SIAH is expressed in 13 human cancer cell lines examined. SIAH is expressed in proliferating cells and its expression is independent of K-RAS activation. D. Both siah1 and siah2 transcripts are expressed in human pancreatic cells. RT-PCR was performed on a panel of human pancreatic cells that included normal epithelial cells (HPDE6), tumor cells (BxPC3), and cancer cells (Panc-1 and MiaPaCa). The relative levels of siah1 and siah2 transcripts in these cells were estimated semiquantitatively by diluting the cDNA templates serially. GAPDH transcript is used as a positive control.
processes from neuronal differentiation, apoptosis, stress response, protein folding to tumor suppression as well as signaling mediated by key signaling molecules including RAS, β-catenin, APC, p53, and NUMB. SINA/SIAHs have been found to interact with and/or degrade more than 28 distinct partners/substrates, most likely through a unique substrate-recognition degron motif (27–29). However, none of the SIAH-interacting proteins are known to play a role in the RAS pathway (25, 30–32). Despite the interest in SIAH-mediated proteolysis in mammalian cells, the role of SIAH in RAS signaling transduction or RAS-mediated oncogenesis remains to be defined.

Activation of the RAS/RAF/MEK/ERK signaling pathway is a major driving force in cell proliferation, transformation, and tumorigenesis (8, 9). Therefore, determining whether, similar to that in Drosophila, mammalian SIAH is required for proper transmission of RAS signal and, furthermore, whether SIAH-dependent proteolysis is required for RAS-mediated tumorigenesis are important areas of investigation. In this report, we show that SIAH is required for proper RAS signal transduction. Furthermore, inhibiting SIAH function reduces ERK signaling and blocks RAS-dependent cell transformation and human pancreatic tumor growth in vitro and in vivo. Thus, SIAH may serve as a suitable and novel anti-RAS therapeutic target in the treatment of human pancreatic cancer.

Materials and Methods

Cell culture. Human pancreatic cell lines were purchased from American Type Culture Collection and maintained according to standard procedures.

Reverse transcription-PCR. Qiagen RNeasy Kit was used to isolate mRNA from human cells (Qiagen). The First-Strand cDNA was reverse transcribed with AMV-RT (First-Strand cDNA Synthesis Kit for reverse transcription PCR, Roche) before PCR using gene-specific primer with Taq polymerase (Roche). siah1/2 specific primers were used for PCR synthesis.

Figure 2. SIAH1/2 PD but not SIAH1/2 WT suppresses oncogenic RAS-mediated foci formation in rat fibroblasts. A to C, SIAH1/2 WT, SIAH1/2 PD, and pcDNA vector were transfected alone or together with activated H-RasV12 in Rat-1 fibroblasts to examine whether RAS-mediated foci formation could be inhibited by the proteolysis-deficient mutant SIAHs. Transfections using single plasmid (pcDNA3 vector, pcDNA3-siah1/2 WT, or pcDNA3-siah1/2 PD) were used as negative controls. No foci were generated in these controls. Three representative 100-mm tissue culture plates (a1, b1, and c1); columns, mean of the experimental results (a2, b2, and c2). A and B, expression of human SIAH1/2 PD dramatically reduced the number of foci induced by oncogenic H-RasV12 when compared with cells transfected with H-RasV12 alone. C, SIAH1/2 WT was cotransfected with H-RasV12. Expression of human SIAH1/2 WT does not affect RAS-mediated foci formation whereas SIAH1/2 PD dramatically suppresses RAS-mediated foci formation.
by Taq polymerase (Roche). The 5’- and 3’-primers for siah1 PCR were 5’-ATGAGCCGTGCAGCTGCA-3’ and 5’-GAGACTGGAAGAGCGAGGTCAGCTC-3’. The 5’- and 3’-primers for siah2 PCR were 5’-GCCATCTCGTCGTGGC-3’ and 5’-ACCAATATGGAGAGGACCGGAGAA-3’. Two-step PCR was used to synthesize the full-length siah1,2 primers using transprimers 5’-TCGCAGTCTTCCTGACTGTTAGCC-3’ and 5’-GGCCGAGG- AACACATAGTCAAAG primer with Eco ACTCGAAGAGCGAGGTCAGCTC-3’. 5’-GGAGAGCCGAGGACTGAGGTCAGCTC-3’ and 5’-GGAGAGCCGAGGACTGAGGTCAGCTC-3’ for siah-1, 5’-GAGCTGACCAGCTCTGGCTATGTGCTGTTTTTT. 3’-POLYOMA tag. \(H\)-RASV12 alone (positive control) or SIAH1/2 foci were stained with 0.2% Crystal Violet in 10% ethanol. As controls, the cells were fixed in 10% methanol and 10% acetic acid and stained with H&E, von Willebrand factor and vimentin. Regulated Proteolysis in the RAS Signaling Pathway

**Expression of human SIAH1,2 WT/PD proteins.** High-fidelity PCR was carried out using the following primers: (a) siah1,2-POLYOMA-tagged primer with EcoRI site: 5’-GGCCGAGGACTGAGGTCAGCTC-3’ and siah1,2 primer with XhoI site: 5’-GGGCTGACGTCAGTGTGTTTTTT. 3’-POLYOMA tag. \(H\)-RASV12 alone (positive control) or SIAH1/2 foci were stained with 0.2% Crystal Violet in 10% ethanol. As controls, the cells were fixed in 10% methanol and 10% acetic acid and stained with H&E, von Willebrand factor and vimentin. Regulated Proteolysis in the RAS Signaling Pathway

**Generation of human SIAH1,2 WT/PD constructs.** The human SIAH1,2 WT/PD cDNA fragments were cloned into the lentiviral vector pHR-SIN-BX-IRES-Em or pHR-SIN-BX-IRES-SIAH1/2 as described (37). Control and SIAH2 PD transgene using primers 5’-GCCGCTAGCTGCAATGTGTTTTTT. 3’-POLYOMA tag.

**Results**

SIAH is expressed in proliferating human cells. Oncogenic K-RAS is detected in the vast majority of pancreatic cancers

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**References**

1. SIAH1/2 PD viruses. Multiple attenuated Lentiviral vector was used to deliver siah1,2 shRNA constructs and SIAH1,2 PD into human cancer cells (34). The three-component system was used to generate the Lentiviruses following standard procedures (35, 36). For shRNA knockdown, we employed the MISSION Lentiviral mediated gene-specific short hairpin RNA (shRNA) system (37). Control and siah1,2 specific shRNA knockdown constructs were produced by Sigma. High-titer Lentiviruses were produced using FuGene (Roche) transfection kit as described (34, 36).
Thus, it is likely that SIAH-1 and SIAH-2 are both expressed in cells. To address this question, we measured the SIAH-2 (Supplementary Fig. S1), we could not differentiate whether and tumorigenesis (Fig. 1; Supplementary Fig. S2).

Because SINA is a known downstream component of the RAS signaling pathway and is thought to be an early event in pancreatic tumorigenesis (13, 39), the roles of mammalian SIAH in the RAS pathway are not well defined. We first developed four mAbs against Drosophila SINA, and tested for cross-reactivity to human SIAHs. Indeed, these anti-SINA mAbs were specific in recognizing the SINA/SIAH family of E3 ligases, as confirmed by Western blot and immunohistochemistry (Supplementary Table S1; Supplementary Fig. S1). Using the anti-SINA/SIAH mAbs, we examined the SIAH expression and localization in normal tissues, human pancreatic cancer cells, and tumor specimens. SIAH expression was detected in all human cancer cell lines and tumor tissues as well as in normal proliferating cells such as skin follicles, kidney, gut, and the germinai center (Fig. 1; Supplementary Fig. S2). The SIAH expression pattern was predominantly nuclear in proliferating cells regardless of the transformation status (Fig. 1B). In contrast, SIAH expression was undetectable in normal nonproliferating human tissues (Fig. 1A), indicating a strong correlation between increased SIAH-dependent proteolysis and active cell proliferation, consistent with its roles in modulating RAS-mediated cell proliferation in normal development and tumorigenesis (Fig. 1; Supplementary Fig. S2).

Because the anti-SINA mAbs recognize both human SIAH-1 and SIAH-2 (Supplementary Fig. S1), we could not differentiate whether one or both SIAH orthologues were up-regulated in proliferating cells. To address this question, we measured the siah1/siah2 transcript levels in human pancreatic cells. Semiquantitative RT-PCR was performed using siah1/siah2-specific primers to amplify mRNA isolated from either normal epithelial cells (HPDE6), tumor cells with wild-type RAS (BxPC-3), or cancer cells with oncogenic RAS (Panc-1 and MiaPaCa). Despite their drastic differences in tumorigenic potential, both siah1 and siah2 mRNA transcripts were expressed at similar levels in these proliferating pancreatic cells (Fig. 1D).

Thus, it is likely that SIAH-1 and SIAH-2 are both expressed in normal and neoplastic proliferating cells.

Expression of SIAHPD blocks H-RASV12-mediated focus formation in Rat-1 fibroblasts. Because SINA is a known downstream component of the Drosophila RAS signaling pathway and virtually all human pancreatic cancers contain hyperactivating K-RAS mutations, we next asked whether modulating SIAH activity might provide a means for suppressing RAS-mediated neoplastic transformation/tumorigenesis. It is known that SINA/SIAH function as a dimer (25, 40). The use of dominant-negative mutants can be an effective strategy to inhibit the endogenous protein that are known to dimerize (41). Toward this end, we constructed mutant SIAH1/2 proteins by introducing two point mutations (2 Cys to Ser) in the conserved RING domains. These SIAH mutations inactivate the E3 ligase activity and block self-ubiquitination and substrate degradation (Supplementary Fig. S3). Because these mutant SIAHs bind substrates but cannot degrade them, we termed the mutant forms of SIAH proteolysis-deficient (SIAHPD). Mutant SIAH PD can bind to wild-type SIAH-2 (SIAHWT), and the binding of mutant SIAHPD to SIAHWT adversely interferes with SIAH2 activity and substrate ubiquitination (Supplementary Fig. S3). Thus, the SIAH PD mutants function as dominant-negatives and are useful tools to dissect the roles of SIAH1/2 in RAS signal transduction.

We first sought to determine whether mammalian SIAH functions downstream of RAS and participates in RAS-dependent transformation. Toward this end, SIAHWT or SIAHPD was expressed in Rat-1 fibroblasts under the control of the cytomegalovirus promoter in combination with oncogenic H-RAS (H-RASV12), and the effects on RAS-mediated focus formation were monitored. Our results showed that SIAH PD expression dramatically decreased the number of foci induced by H-RAS V12, as compared with positive controls transfected with H-RASV12 alone (Fig. 24 and B). In contrast, SIAHWT expression did not alter the number of foci induced by H-RAS V12 under the same conditions, suggesting that SIAH WT expression alone is not sufficient to drive focus formation and cellular transformation (Fig. 2C). These results indicate that blocking SIAH function inhibits RAS-mediated transformation in fibroblasts.

SIAH is required for anchorage-independent tumor growth of human pancreatic cancer cells in soft agar. We next sought to determine whether SIAH participates in RAS-mediated anchorage-independent growth and malignant transformation of human pancreatic cancer cells. We used either the pcDNA3 or pLentiviral vector to deliver SIAH PD into MiaPaCa and Panc-1 cells, and examined whether SIAH PD expression could inhibit the anchorage-independent tumor growth of these cancer cells in soft agar. Indeed, the colony formation of both MiaPaCa-SIAH PD and Panc-1-SIAH PD cells were significantly less than those of control cells (Fig. 3b2 and b3). Thus, these results support the notion that SIAH PD expression blocks the RAS-mediated anchorage-independent tumor growth of human pancreatic cancer cells.

To substantiate the above observations, we used an alternative approach to disrupt endogenous SIAH function by using interfering short hairpin shRNA to further delineate SIAH function in the mammalian RAS pathway. Employing the MISSION pLentiviral-shRNA system to silence siah1/2 expression (37), we achieved an ~2- to 3-fold knockdown of siah1/2 transcripts in human cancer cells using the siah1/2-specific shRNA constructs (nos. 6 and 8), as shown by semiquantitative RT-PCR, Western blot analyses, and

Figure 3. SIAH deficiency compromises anchorage-independent tumor growth in soft agar. A, the MISSION pLentiviral-mediated gene-specific shRNA knockdown system was effective in knocking down endogenous siah-2 transcripts in Panc-1 cells. a1, RT-PCR was used to semiquantitatively estimate the extent of the siah-2 knockdown as 2- to 3-fold for shRNA constructs nos. 6 and 8. a2, to show the efficacy of shRNA knockdown, a MiaPaCa stable line (line 7) expressing the FLAG-tagged SIAH WT at a high level was treated with pLenti-shRNA viruses carrying the siah-2 knockdown constructs. Multiple pLenti-shRNA constructs (nos. 6, 7, and 8) were effective in knocking down the exogenously expressed SIAH. a3, the pLenti-shRNA constructs (nos. 6 and 8) were also effective in knocking down endogenously expressed SIAHs. Note that phospho-ERK activity was markedly down-regulated in siah-2 knockdown cells (nos. 6 and 8). a4, mouse anti–SIAH-2 mAb purchased from Sigma was used to examine endogenous SIAH-2 expression in Panc-1 cells by Western blots. Note that SIAH2 expression is reduced in siah-2 knockdown cells (no. 6) and phospho-ERK activity was markedly down-regulated in siah-2 knockdown cells (no. 6) as well as SIAH2-expressing cells. a5, the pLenti-shRNA constructs (nos. 6 and 8) were also effective in reducing the anti–SIAH2 MEK5/6 staining in Panc-1 cells, showing the mAb specificity against SIAHs. B, RAS-mediated anchorage-independent growth of uninfected Panc-1 cells and Panc-1 cells infected with either pLenti-shRNA siah-2 knockdown viruses (nos. 6, 7, or 8) separately, pLenti-shRNA control and pLenti-GFP viruses, or pLenti-SIAH WT viruses were examined using soft agar assays. b1, two thousand infected Panc-1 cells were plated into six-well plates in triplicate. Columns, mean experimental results shown above the representative Panc-1 colony images captured at days 1, 7, and 14. Note that pLenti-shRNA–mediated knockdown of siah-2 (no. 8) was effective in blocking Panc-1 colony formation in soft agar. b2, two hundred infected Panc-1 cells were plated into 24-well plates in triplicate. Every colony formed at day 14 was counted; columns, mean of the experimental results is shown above the colony images. Note that SIAH-deficiency either through shRNA knockdown (no. 6) or stable SIAH PD expression is equally effective in blocking colony formation in soft agar. b3, two thousand MiaPaCa cells and MiaPaCa stable lines expressing either pcDNA3 or FLAG-tagged SIAH WT were plated in triplicate into six-well plates. Columns, mean of experimental results is shown above the representative MiaPaCa colony images captured. Note that MiaPaCa cells stably expressing SIAH WT are defective in forming colonies in soft agar.
immunohistochemical staining (Fig. 3A). Furthermore, to show the knockdown efficacy of these *siah*-shRNA constructs, we used a MiaPaCa-SIAH PD stable line (Supplementary Fig. S4A1, line 7) and showed that the exogenous N-terminal tagged FLAG-SIAH PD was specifically knocked down by a number of pLenti-shRNA constructs against the *siah*-2 gene (Fig. 3A2).

Using these shRNA constructs, we next determined whether a reduction in *siah*-2 expression affects anchorage-independent cell growth and tumorigenesis of human pancreatic cancer cells. Similar to results obtained using SIAH PD, the *siah*-2 transcript knockdown markedly reduced colony formation of these cancer cells in soft agar (Fig. 3b1 and b2). Interestingly, in analyzing the effects of either SIAH PD expression or *siah*-2 knockdown on Ras signaling, we observed that the phospho-ERK signal was significantly reduced in these cancer cells with decreased SIAH function (Fig. 3A3 and A4). The results from these experiments using both shRNA-mediated knockdown and SIAH PD suggest that SIAH is required for Ras-mediated malignant transformation and anchorage-independent tumor growth of human pancreatic cancer cells in soft agar. Furthermore, reduction of ERK signaling upon SIAH inhibition suggests that SIAH2 or SIAH2 substrates may be part of a regulatory feedback loop that is involved in modulating Ras/Raf/MEK/Mapk signaling in human pancreatic cancer cells.

Expression of SIAH1 PD abolishes human pancreatic tumor formation in athymic nude mice. Oncogenic K-RAS mutations are known to drive pancreatic tumor initiation and cancer progression (13). We next examined whether SIAH participates in K-RAS–mediated tumorigenesis in vivo. First, we asked whether blocking SIAH function could inhibit K-RAS–mediated pancreatic tumor formation of MiaPaCa and Panc-1 cells (representing highly aggressive human pancreatic adenocarcinomas) in athymic nude mice. SIAH1 PD or vector control was introduced into these cancer cells using pcDNA3 transfection or pLentiviral infection, and the manipulated cancer cells were implanted in nude mice. Subsequently, the tumor formation of these cancer cells was monitored to determine the efficacy of SIAH PD molecules in blocking Ras-mediated pancreatic tumorigenesis in vivo.

Stable MiaPaCa-SIAH1 PD cell lines were cloned, amplified, and checked for SIAH PD expression (Supplementary Fig. S4A). One million MiaPaCa cells expressing either pcDNA3 or SIAH1 PD were then implanted s.c. into athymic nude mice. Tumor formation was monitored over a 7-week period, as shown by the growth curve in Fig. 4B. Fifty-five of 60 injections of 10^6 MiaPaCa-pcDNA3 cells into 30 nude mice generated large tumors within 6 weeks. In contrast, none of the 110 injections of 10^6 MiaPaCa-SIAH1 PD cells into 55 nude mice generated any sizable tumors (Fig. 4A–C). Thus, the expression of SIAH1 PD in MiaPaCa cells severely impeded the formation of pancreatic tumors in nude mice (Fig. 4). These MiaPaCa tumors expressing the mutant forms of SIAH1 PD did, however, exhibit a slight amount of growth beyond 10 weeks, indicating that these SIAH1 PD-expressing MiaPaCa cells, although growth-defective, were still viable in vivo (Supplementary Fig. S4C). Because SIAHs form homodimers and heterodimers (40), and both SIAH PD and SIAH PD have similar antitumor effects for MiaPaCa cells in nude mice, it is conceivable that SIAH PD may interfere with both endogenous SIAH1 and SIAH2 functions. Thus, we focused on characterizing the SIAH PD phenotypes in subsequent studies.

For analysis of in vivo tumor formation by another independent human pancreatic cancer cell line, an HIV-based pLentivirus that allows for the coexpression of SIAH PD and eGFP in a bicistronic fashion under the control of the SFFV promoter was used to infect Panc-1 cells (34, 36). Almost 100% of the cells were infected as indicated by the eGFP marker (Fig. 5B). Both heterogeneous populations of Panc-1 cells infected with high-titer pLenti-eGFP control viruses or pLenti-SIAH PD-eGFP viruses, as well as multiple stable Panc-1-GFP or Panc-1-SIAH PD-eGFP cell lines were established. The SIAH PD expression levels in the Panc-1-SIAH PD stable cell lines are shown (Fig. 6b). Panc-1-SIAH PD-eGFP stable line 2 and 3. Tumorigenesis was examined in nude mice using both heterogeneously infected Panc-1 cells as well as the Panc-1 stable lines. One million Panc-1-eGFP and Panc-1-SIAH PD-eGFP cells were implanted s.c. into athymic nude mice, and tumor formation was monitored over 3 months. The eGFP expression slowed the Panc-1 tumor growth rate due to some GFP toxicity (Fig. 5b1).

Nevertheless, a remarkable blockage in pancreatic tumor growth was observed in Panc-1-SIAH PD cells, even following extended tumor incubation periods of up to 4 months in nude mice (Supplementary Fig. S4D). Thus, these results clearly show that blocking SIAH function dramatically reduces the tumor formation by two highly aggressive pancreatic cancer cell lines in vivo (Figs. 4 and 5).

To better understand SIAH PD–mediated tumor suppression at the cellular level, a careful immunohistochemical analysis of the large and small tumors resected from the nude mice was performed to assess changes in cellular morphology, tumor architecture, and angiogenesis. The control and SIAH PD–expressing MiaPaCa and Panc-1 tumors were fixed, sectioned, and stained with H&E, anti–SIAH 24E6, and anti-vWF antibodies stain both large and small tumors.

Figure 4. Human SIAH PD blocks MiaPaCa tumor growth in athymic nude mice. One million MiaPaCa cells stably expressing pcDNA3, human SIAH PD, or Polyoma-tagged (SIAH-1-PD), or human SIAH PD–FLAG–tagged (SIAH-2-PD) were injected s.c. into the right and left flanks of 5-week-old male athymic nude mice (n = 10–15 mice per construct/group). Tumor growth was measured every 3 d until 7 wk after injection. A, five representative mice in each injection group are shown, with the tumors that developed at both injection sites shown below the corresponding mice. Blocking SIAH function dramatically suppresses the tumor growth of human pancreatic cancer cells in athymic nude mice when compared with MiaPaCa cells transfected with pcDNA3 vector alone. Graphs displaying the tumor growth rates (B) and average tumor mass following resection of the human pancreatic cancers (C) carrying pcDNA or the SIAH PD transgene in nude mice. D, histology was performed on the large and small tumors originating from the MiaPaCa-pcDNA and MiaPaCa-SIAH PD cells. d1 to d4 and d9 to d12. H&E was used to view tumor tissue morphology of the human pancreatic cancer cells resected from the nude mice. Human cancer cells are clearly visible because they are bigger in size and have bigger nuclei than mouse cells, and stained with anti–SIAH 24E6 mAb. d5 to d8, anti-SIAH mAb (24E6) stains human cells but not surrounding mouse cells. d13 to d16, anti-vWF antibodies stain both large and small tumors.
MiaPaCa-SIAH1/2 from MiaPaCa-pcDNA3 cells (Supplementary Fig. S4) deficiency results in a significant reduction in ERK signaling provided in the medium. Thus, SIAH may be a necessary licensing factor for tumorigenesis provided pivotal insights into RAS signal transduction cascades. SINA is the most downstream component identified in the Drosophila RAS pathway (23). As a logical extension of our Drosophila studies, we asked whether human SIAH E3 ligases might similarly serve as a downstream gatekeeper for RAS signal transduction in cancer. SIAH is expressed in proliferating cells (both normal and malignant cells) but not in nonproliferating cells (Fig. 1; Supplementary Fig. S2). Because the RAS pathway is active in proliferating cells, it is conceivable that SIAH expression may be required for transmitting the active RAS signal which drives cell proliferation. Importantly, we observed that blocking SIAH function by SIAH<sup>PD</sup> suppresses RAS-mediated fibroblast transformation, anchorage-independent tumor growth in soft agar, and pancreatic cancer growth in nude mice (Figs. 3–5). Similarly, shRNA-mediated <em>siah</em> mRNA knockdown also reduced the anchorage-independent growth of pancreatic cancer cells in soft agar (Fig. 3B). Interestingly, SIAH-deficiency in two pancreatic cancer cell lines containing oncogenic K-RAS, MiaPaCa, and Panc-1 resulted in a significant reduction in ERK signaling (Figs. 3A3 and A4 and 6A and B). Thus, the inhibitory effects of reduced SIAH function on the growth of pancreatic tumors, either through SIAH<sup>PD</sup> expression or shRNA-mediated <em>siah</em> knockdown, may occur through effects on upstream components of RAS/MEK/ERK signaling as a result of a SIAH- or SIAH substrate–regulated feedback loop mechanism (Fig. 6C).

Discussion

Oncogenic RAS mutations promote neoplastic transformation and tumorigenesis (10, 13). To date, direct targeting of hyper-activated K-RAS has shown rather limited therapeutic efficacy in the treatment of human pancreatic cancer (8). As such, additional targets and therapies are urgently needed. Here, we show that a downstream component of the mammalian RAS signaling pathway, SIAH, plays an essential role in RAS-mediated transformation and tumorigenesis. Thus, as an enzyme, SIAH E3 ligase may serve as an ideal target in the development of novel therapeutics for the treatment of pancreatic cancers in which aberrant RAS signaling is an early signaling event and a major driving force in neoplastic transformation and pancreatic tumorigenesis.

Figure 5. Human SIAH<sup>PD</sup> blocks Panc-1 tumor growth in athymic nude mice. One million Panc-1 cells either heterogeneously infected or stably expressing eGFP or human SIAH<sup>PD-5</sup>–FLAG–tagged-eGFP (SIAH2-PD) were injected s.c. into the right and left flanks of 5-week-old male athymic nude mice (n = 10 mice per construct/group). One million uninfected Panc-1 cells were injected as controls. A, five representative mice in each injection group, with the tumors that developed at both injection sites shown below the corresponding mice. Blocking SIAH function dramatically suppresses the tumor growth of Panc-1 cells in nude mice when compared with uninfected Panc-1 and Panc-1 cells expressing eGFP. B, tumor growth, tumor volume, and weights post-surgery (day 1). Graph displaying the tumor growth rates of uninfected Panc-1 cells or Panc-1 expressing GFP or SIAH<sup>PD-5</sup>–GFP transgene stably or heterogeneously in nude mice. Note that the presence of GFP slowed tumor growth due to some GFP toxicity. A2, the GFP and bright-field images of plentiviral–infected Panc-1 cells expressing either GFP or SIAH<sup>PD-5</sup>–GFP are shown at days 2 to 7 postinfection. The pLentiviral infection rate was close to 100% in the heterogeneous Panc-1 population. Graph displaying average volume (b3) and mass (b4) of these tumors following surgical resection. C, histology was performed on the large and small tumors originating from the Panc-1, Panc-1-SIAH2-5F–stable line and Panc-1 heterogeneously infected with pLenti-GFP or plentiv-SIAH2-5F–GFP viruses. c1 to c8, H&E was used to view the tissue morphology of Panc-1 tumors resected from the nude mice. Human cells are bigger in size and have bigger nuclei than mouse cells. c9 to c16, anti-SIAH mAb (24E6) stains human cells but not the surrounding mouse cells. c17 to c24, anti-vWF antibodies stain both large and small tumors.
Figure 6. SIAH-deficiency reduces MAPK signaling and a schematic model summarizing the proposed role of SIAH-dependent proteolysis in the mammalian RAS signal transduction pathway. A, a panel of human pancreatic cells was mock-infected or infected with pLenti-GFP or pLenti-SIAH2 PD viruses. Note that phospho-ERK signaling was also down-regulated in Panc-1 and MiaPaCa cells with oncogenic RAS, whereas phospho-ERK signaling was not significantly altered in normal pancreatic epithelial cells (HPDE6) or tumor cells with wild-type RAS (BxPC-3). B, the signaling events downstream of RAS were examined by Western blots in stable lines of three pancreatic tumor/cancer cells. The expression of SIAH2 PD dramatically suppressed MAPK signaling and phospho-ERK expression in both MiaPaCa and Panc-1 stable lines with constitutively activated RAS signal although the MAPK signaling and phospho-ERK expression remained unchanged in BxPC-3 cells with wild-type RAS. C, the human RAS signal transduction pathway is highly conserved with the RAS pathway described in model organisms and contains the same downstream signaling modules such as RAS, RAF, MEK, MAPK, and the ETS family of transcription factors. c1, the RAS signal transduction pathway in Drosophila. SINA is the most downstream component identified in the RAS pathway and it is absolutely required for transmission of activated RAS/RAF/MAPK/ETS signals in the fly. c2, our proposed model in which SIAH is a downstream component of the mammalian RAS signal transduction pathway. SIAH-dependent proteolysis is required for proper mammalian RAS signaling. SIAH-deficiency might affect MAPK signaling through a negative feedback loop control mechanism. By blocking SIAH function, we can inhibit RAS signaling and RAS-dependent transformation and tumorigenesis in mammalian systems.
in a panel of normal and malignant human pancreatic cells followed by Western blot analysis of components of the RAS/RAF/MEK/ERK cascade did, however, reveal that ERK signaling in cells with wild-type RAS (HPDE6 and BxPC-3) was less affected than those with oncogenic RAS (Panc-1 and MiaPaCa; Fig. 6a and B). The differential requirement for SIAH in RAS signaling in cells expressing wild-type RAS versus oncogenic RAS could be due to rewiring of cancer signaling pathways induced by hyperactivated K-RAS and a higher dependency on increased SIAH activity for transmitting proper RAS signaling in cancer cells. The roles of SIAH in RAS signaling and RAS-mediated transformation/tumorigenesis are still open areas of investigation. Human pancreatic cancer cells expressing SIAH2PD did exhibit a markedly reduced capacity to form tumors in both soft agar and nude mouse tumor assays (Figs. 3–5), although there were no dramatic inhibitory effects on cell proliferation and cell cycle progression under tissue culture conditions (Supplementary Fig. S4). Thus, SIAH-dependent proteolysis may play a role in modulating cancer cell behavior and tumor microenvironment in a manner essential for sustaining tumor growth.

Using dominant-negative constructs to block SIAH function may have other unintended side effects. SIAH forms homodimers as well as heterodimers (40), and for proteins that form dimers, the dominant-negative approach is a useful strategy for blocking endogenous protein function (41). Indeed, the ERK pathway was specifically affected in a similar fashion both when shRNA-mediated siah-2 knockdown and expression of SIAH2PD were used to inhibit endogenous SIAH function, indicating the specificity and efficacy of both agents in blocking RAS signaling in human cancer cells (Figs. 3a2–a5 and 6a and B). Our results, in which inhibiting SIAH significantly impairs human pancreatic tumorigenesis in nude mice, provides an important step forward in identifying novel therapeutics of blocking RAS signaling by targeting the downstream SIAH-dependent proteolytic machinery. Moreover, by targeting SIAH specifically to inhibit tumor formation, one might avoid complications engendered by other agents that lead to a general inhibition of cell proliferation or proteosome function systemically.

Pancreatic cancer is the fifth leading cause of cancer-related death with a median survival of only 6 months (13, 14); however, conventional therapies have little effect on the course of this disease (13). As such, there is an urgent need to understand the molecular events underlying the cause and progression of pancreatic cancers and develop novel therapeutic agents to treat this devastating form of cancer. The RAS and ubiquitin-mediated proteolysis pathways have a demonstrated importance in neoplastic transformation and tumorigenesis (8, 9, 43–45). Small molecules that block proteosome activity have shown some clinical promise as novel antineoplastic agents in treating patients with relapsed and refractory multiple myeloma; however, the systemic toxicity due to global inhibition of proteosome function is of significant concern (46, 47). Thus, the development of specific inhibitors that selectively target key E3 ligases, such as MDM2 and SIAH, thereby inhibiting important signaling pathways dysregulated in human cancer, would be highly desirable and effective (43, 44, 48).

How regulated proteolysis in the RAS pathway contributes to neoplastic transformation/tumorigenesis remains to be elucidated. Here, we show that RAS signal transduction depends on proper SIAH function in cancer cells. (a) SIAH is expressed in dividing cells whereas its expression is absent in nondividing cells. (b) Blocking SIAH function through either the dominant-negative SIAH1/2 or shRNA-mediated siah-2 knockdown inhibits RAS-mediated foci formation in fibroblasts, anchorage-independent tumor growth in soft agar, and human pancreatic tumor formation in nude mice. (c) Although SIAH is a downstream component of RAS signaling, inhibition of SIAH function results in a reduction in ERK signaling. Thus, SIAH may be a necessary downstream transducer of activated RAS signaling in mammalian cells and that SIAH-mediated proteolysis is required for RAS-dependent neoplastic transformation/tumorigenesis. Furthermore, as the inhibition of SIAH enzymatic activity impaired the ability of highly aggressive human pancreatic cancer cells to form tumors in vivo, this raises an exciting possibility that targeting of SIAH may be a viable anticancer therapeutic option. The next generation of small molecules aimed at targeting SIAH-mediated proteolysis in RAS signaling pathways may yield highly selective therapeutics that could halt the progression of human pancreatic cancer and save the lives of those with this devastating disease.

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