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Impeded Nedd4-1-Mediated Ras Degradation Underlies Ras-Driven Tumorigenesis

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SUMMARY

RAS genes are among the most frequently mutated proto-oncogenes in cancer. However, how Ras stability is regulated remains largely unknown. Here, we report a regulatory loop involving the E3 ligase Nedd4-1, Ras, and PTEN. We found that Ras signaling stimulates the expression of Nedd4-1, which in turn acts as an E3 ubiquitin ligase that regulates Ras levels. Importantly, Ras activation, either by oncogenic mutations or by epidermal growth factor (EGF) signaling, prevents Nedd4-1-mediated Ras ubiquitination. This leads to Ras-induced Nedd4-1 overexpression, and subsequent degradation of the tumor suppressor PTEN in both human cancer samples and cancer cells. Our study thus unravels the molecular mechanisms underlying the interplay of Ras, Nedd4-1, and PTEN and suggests a basis for the high prevalence of Ras-activating mutations and EGF hypersignaling in cancer.

INTRODUCTION

Ras proteins (K-Ras, H-Ras, and N-Ras) are small guanosine triphosphatases (GTPases) that act as key molecular switches in regulating a wide range of cellular processes, especially in controlling cell proliferation, transformation, differentiation, and survival (Ahearn et al., 2012; Malumbres and Barbacid, 2003). Their activities are tightly controlled by cycling between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound states (Ahearn et al., 2012). Serving as a signaling hub, Ras proteins are activated by guanine nucleotide exchange factors (GEFs) in response to upstream signaling from binding of various growth factors such as epidermal growth factor (EGF) to their tyrosine kinase receptors, triggering diverse downstream signal cascades (Ahearn et al., 2012; Downward, 2003). The activated GTP-bound form of Ras proteins is converted to the GDP-bound form by their GTPase-activating proteins (GAPs).

Mutations in Ras that predominantly occur at G12, G13, and Q61 result in a persistent GTP-bound state of Ras due to a defect in hydrolysis of GTP to GDP, leading to a constitutive activation of downstream signaling (Buhrman et al., 2010; Scheffzek et al., 1997; Scheidig et al., 1999). Ras mutations are found in about 30% of all human cancers, and up to 90% in pancreas cancer and 50% in colorectal or thyroid cancer, establishing *RAS* genes as the most prominent proto-oncogenes (Bos, 1989; Downward, 2003; Pylayeva-Gupta et al., 2011). In addition to the oncogenic activation by point mutations, Ras signaling can also be aberrantly activated by epidermal growth factor receptor (EGFR) overexpression or mutations, or increased production of autocrine EGF-like factors in tumors (Downward, 2003). For the past 4 decades, intensive efforts have been made to determine the underlying mechanism for regulation of Ras activity and Ras-induced tumorigenesis.

The Nedd4 (neural precursor cell expressed developmentally downregulated protein 4) family ubiquitin ligases belong to the homologous to the E6-AP carboxyl terminus type of E3s and play important roles in tumor progression by affecting various tumorigenesis-related signaling pathways, including transforming growth factor β, EGF, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and tumor necrosis factor α (Chen and Matesic, 2007; Rotin and Kumar, 2009). Nedd4-1 is the founding member of this family. It was originally identified as an E3 ligase for the epithelial sodium channel (ENaC) that regulates ENaC endocytosis and stability (Staub et al., 1996, 2000). Further studies showed that Nedd4-1 could target other different substrates including insulin-like growth factor 1 receptor (IGF1R), vascular endothelial growth factor receptor 2 (VEGF-R2), and phosphatase and tensin homolog (PTEN) for degradation (Chen and Matesic, 2007; Rotin and Kumar, 2009), suggesting that Nedd4-1 may play distinct roles in the regulation of different cellular functions.

Because PTEN is a powerful tumor suppressor that negatively regulates phosphatidylinositol 3-kinase/AKT signaling by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (Song et al., 2012), Nedd4-1 was thus described as an oncoprotein (Wang et al., 2007). However, a later study using cells derived from Nedd4-1 knockout mice embryos indicated that PTEN was not regulated by Nedd4-1 in primary cells (Fouladkou

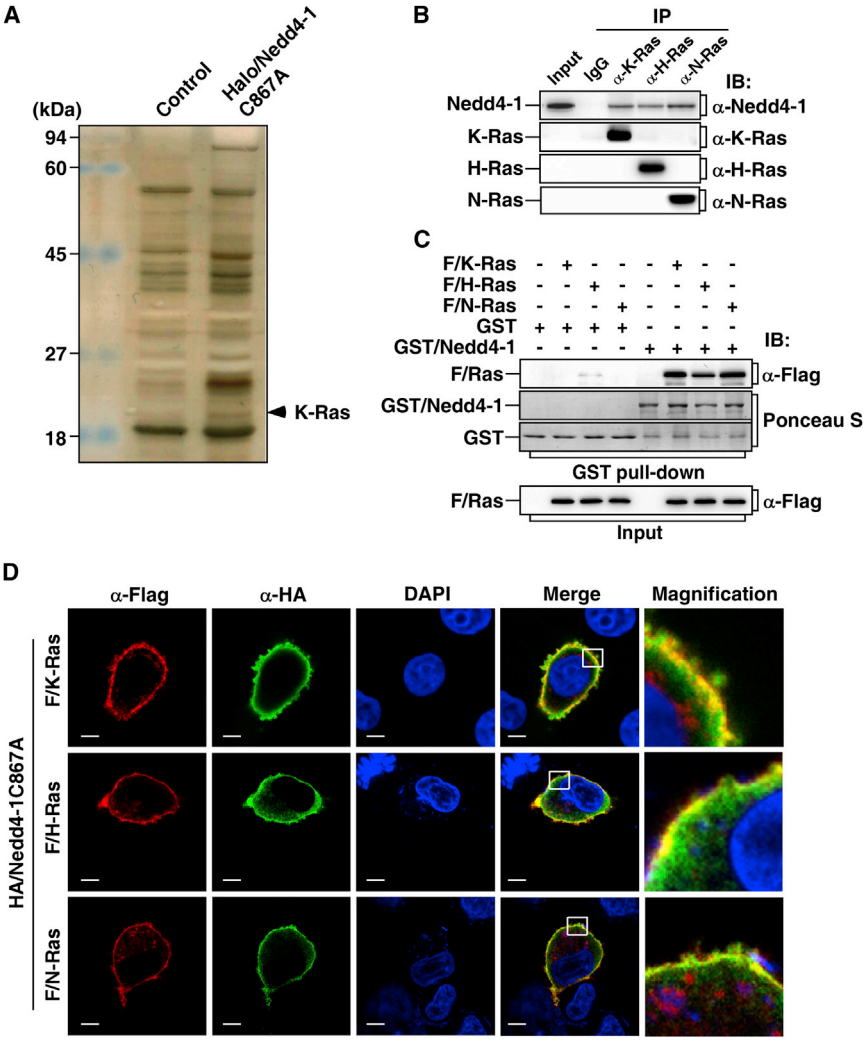

Figure 1. Nedd4-1 Interacts with Ras Proteins

(A) K-Ras associates with Nedd4-1. Halo-tagged Nedd4-1 C867A (Halo/Nedd4-1 C867A) or Halo-tag control transiently expressed in HEK293T cells was purified and applied to SDS-PAGE. The proteins were visualized by silver staining, and indicated spots were analyzed by mass spectrometry.

(B) Interaction of endogenous Nedd4-1 and Ras proteins. Cell lysates from HEK293T were subjected to IP with specific antibodies to K-Ras, H-Ras, or N-Ras followed by immunoblotting (IB) to detect endogenous Nedd4-1.

(C) In vitro interaction between Nedd4-1 and Ras proteins. Bacterially expressed and purified FLAG-tagged K-Ras (F/K-Ras), H-Ras (F/H-Ras), or N-Ras (F/N-Ras) and GST-tagged Nedd4-1 (GST/Nedd4-1) were subjected to GST pull-down assays as indicated. Associated Ras proteins were detected with anti-FLAG. GST and GST/Nedd4-1 were determined by Ponceau S staining.

(D) Nedd4-1 colocalizes with Ras proteins at plasma membrane. HeLa cells were cotransfected with different FLAG-tagged Ras and HA-tagged Nedd4-1 C867A (HA/Nedd4-1 C867A) as indicated. Localization of Ras (red) and Nedd4-1 (green) was detected by immunofluorescence. The nuclei were stained with DAPI (blue). The scale bars indicate 5 μm.

et al., 2008). Interestingly, it was found that phosphorylation of PTEN by tyrosine kinase Rak inhibits the interaction between Nedd4-1 and PTEN (Yim et al., 2009), suggesting that Nedd4-1 may target PTEN only in certain oncogenic contexts such as loss of Rak. In this study, we demonstrate that Nedd4-1 plays a key role in suppressing tumorigenesis, likely by targeting Ras proteins for degradation in normal cells. Importantly, we show that oncogenic activation of Ras evades the Nedd4-1-mediated degradation, leading to an aggravated PTEN degradation by rendering Nedd4-1 overexpressed in cancer cells, presenting a root cause for uncontrolled activation of Ras and potential initiation of tumorigenesis.

RESULTS

Nedd4-1 Interacts with Ras Proteins

To systematically identify binding partners for Nedd4-1, we carried out affinity purification using human embryonic kidney 293T (HEK293T) cells by the HaloTag system (Promega), followed by mass spectrometry analysis to determine the identities of Nedd4-1 binding proteins. Among the detected proteins, the small GTPase K-Ras was identified (Figure 1A). To verify the interaction, we used specific antibodies to immunoprecipitate endogenous K-Ras, H-Ras, or N-Ras from HEK293T cell lysate, and ascertained that endogenous Nedd4-1 was indeed coprecipitated with all three major forms of Ras proteins (Figure 1B), suggesting that Nedd4-1 and Ras proteins interact endogenously.

Next, we performed in vitro GST pull-down assay to examine whether Nedd4-1 and Ras could directly interact. Using bacterially produced FLAG-tagged Ras proteins to incubate with GST or GST/Nedd4-1 beads, we found that GST/Nedd4-1 could pull down all three forms of Ras proteins, indicating a direct interaction between Nedd4-1 and Ras proteins (Figure 1C). Furthermore, we determined that Nedd4-1 and Ras proteins were mainly colocalized at plasma membrane using immunofluorescence assay (Figure 1D).

Nedd4-1 Targets Ras Proteins for Ubiquitination and Degradation

Because Nedd4-1 is an E3 ubiquitin ligase, we asked whether Ras proteins are substrates for Nedd4-1. We first examined effects of Nedd4-1 overexpression on steady-state levels of Ras proteins. Indeed, wild-type (WT) Nedd4-1, but not catalytically inactive mutant Nedd4-1 C867A or its closest family member Nedd4-2, significantly decreased both the endogenous and

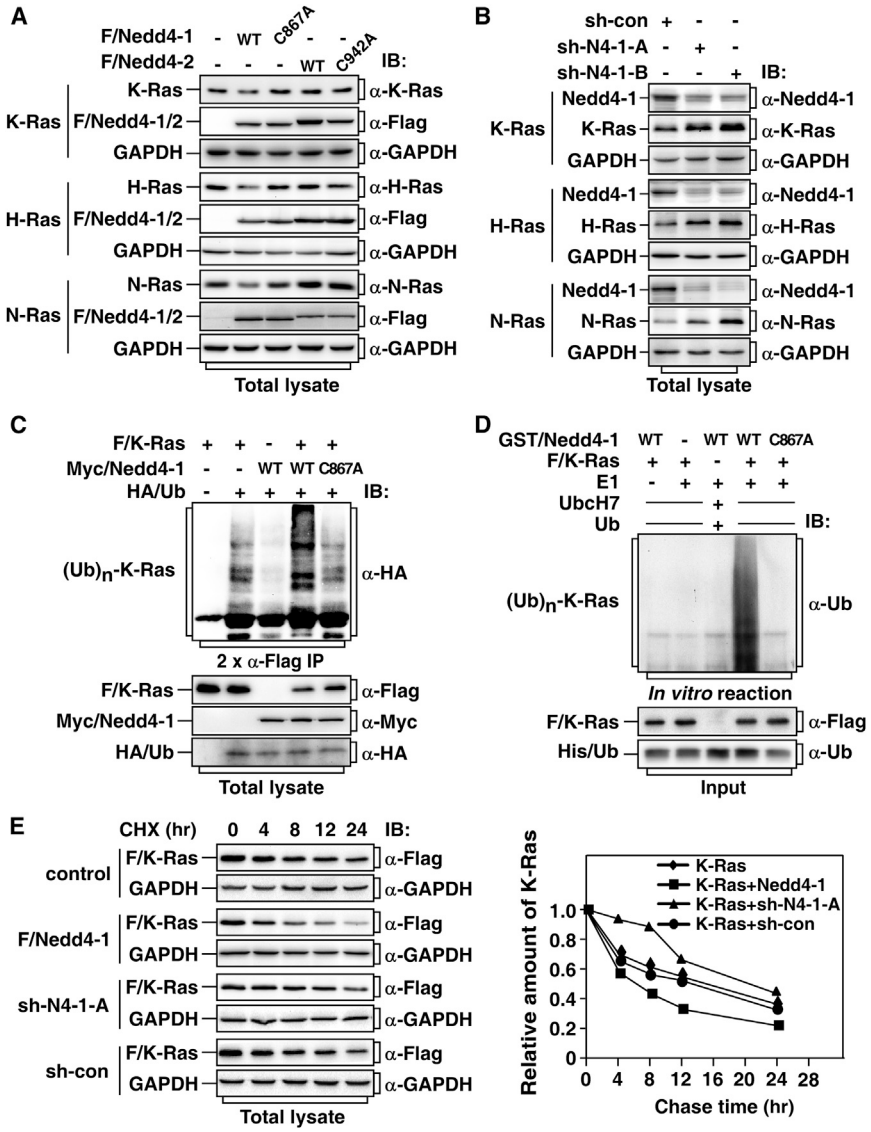

Figure 2. Nedd4-1 Targets Ras Proteins for Ubiquitination and Degradation

(A) Nedd4-1 overexpression decreases endogenous levels of Ras proteins. HEK293T cells were transiently transfected with FLAG-tagged Nedd4-1 (F/Nedd4-1) or Nedd4-2 (F/Nedd4-2), WT or catalytically inactive mutants C867A or C942A as indicated. Total cell lysates were subjected to immunoblotting to determine the protein levels. Levels of GAPDH were used as a loading control.

(B) Knocking down Nedd4-1 increases endogenous Ras protein levels. HEK293T cells were transfected with control shRNA (sh-con) or Nedd4-1 shRNA (sh-N4-1-A or sh-N4-1-B), and the endogenous protein levels of Ras and Nedd4-1 were detected by immunoblotting.

(C) Nedd4-1 promotes ubiquitination of K-Ras. After overnight treatment of 120 μM chloroquine, HEK293T cells transfected with indicated combinations of F/K-Ras, Myc-tagged Nedd4-1 (Myc/Nedd4-1) (WT or C867A), and HA-tagged ubiquitin (HA/Ub) were subjected to anti-FLAG IP, eluted by boiling in 1% SDS, and then reprecipitated with anti-FLAG (2× IP). The ubiquitin-conjugated K-Ras ((Ub)n-K-Ras) was detected with anti-HA.

(D) Direct ubiquitination of K-Ras mediated by Nedd4-1 in vitro. GST/Nedd4-1 (WT or C867A) and F/K-Ras purified from bacteria were subjected to an in vitro ubiquitination assay. Ubiquitinated F/K-Ras was detected with anti-Ub.

(E) Nedd4-1 regulates turnover rates of K-Ras. HEK293T cells were transfected with combinations of F/K-Ras, F/Nedd4-1, control shRNA, and Nedd4-1 shRNA as indicated. The levels of F/K-Ras at different time points after cycloheximide (CHX) treatment were determined by immunoblotting the total cell lysates and quantification using Image Lab software (Bio-Rad) with GAPDH as a loading control. Results plotted are the amounts of F/K-Ras at each time point relative to the level at time 0.

exogenous Ras protein levels (Figures 2A and S1A–S1C), suggesting a specific role of Nedd4-1 in downregulation of Ras protein levels. Accordingly, knocking down Nedd4-1 markedly increased endogenous Ras protein levels (Figures 2B and S1D). In addition, the lysosome inhibitor chloroquine, but not the proteasome inhibitor MG-132, could reverse the decrease of Ras mediated by Nedd4-1, indicating that this downregulation of Ras is likely through the lysosome pathway (Figures S1E and S1F). Furthermore, the effect of Nedd4-1 on downregulating total Ras protein levels was verified in various other cell lines including human cervical cancer HeLa cells, human gastric cancer BGC-823 cells, mouse fibroblast NIH 3T3 cells, and mouse embryonic fibroblast (MEF) cells (Figures S1G–S1J).

Next, we examined whether Nedd4-1 could regulate ubiquitination of Ras proteins. As predicted, overexpression of WT Nedd4-1 but not Nedd4-1 C867A mutant markedly enhanced ubiquitination of all three forms of Ras (Figures 2C and S1K). We also used bacterially expressed FLAG-tagged Ras proteins and GST/Nedd4-1 to perform in vitro ubiquitination assay and found that Nedd4-1 could directly catalyze ubiquitin conjugation of Ras proteins (Figures 2D and S1L), confirming that Ras proteins are direct substrates of Nedd4-1. Accordingly, coexpression of Nedd4-1 significantly decreased the half-lives of Ras proteins, whereas knockdown of Nedd4-1 prolonged the half-lives of Ras proteins (Figures 2E, S1M, and S1N).

Knockdown of Nedd4-1 Induces Cell Transformation and Tumorigenesis

Because Ras small GTPases are well-known proto-oncoproteins, we hypothesized that Nedd4-1 might be involved in controlling tumorigenesis. To test this hypothesis, we first performed soft agar colony-formation assays to examine whether knockdown of Nedd4-1 could induce cell transformation. We used

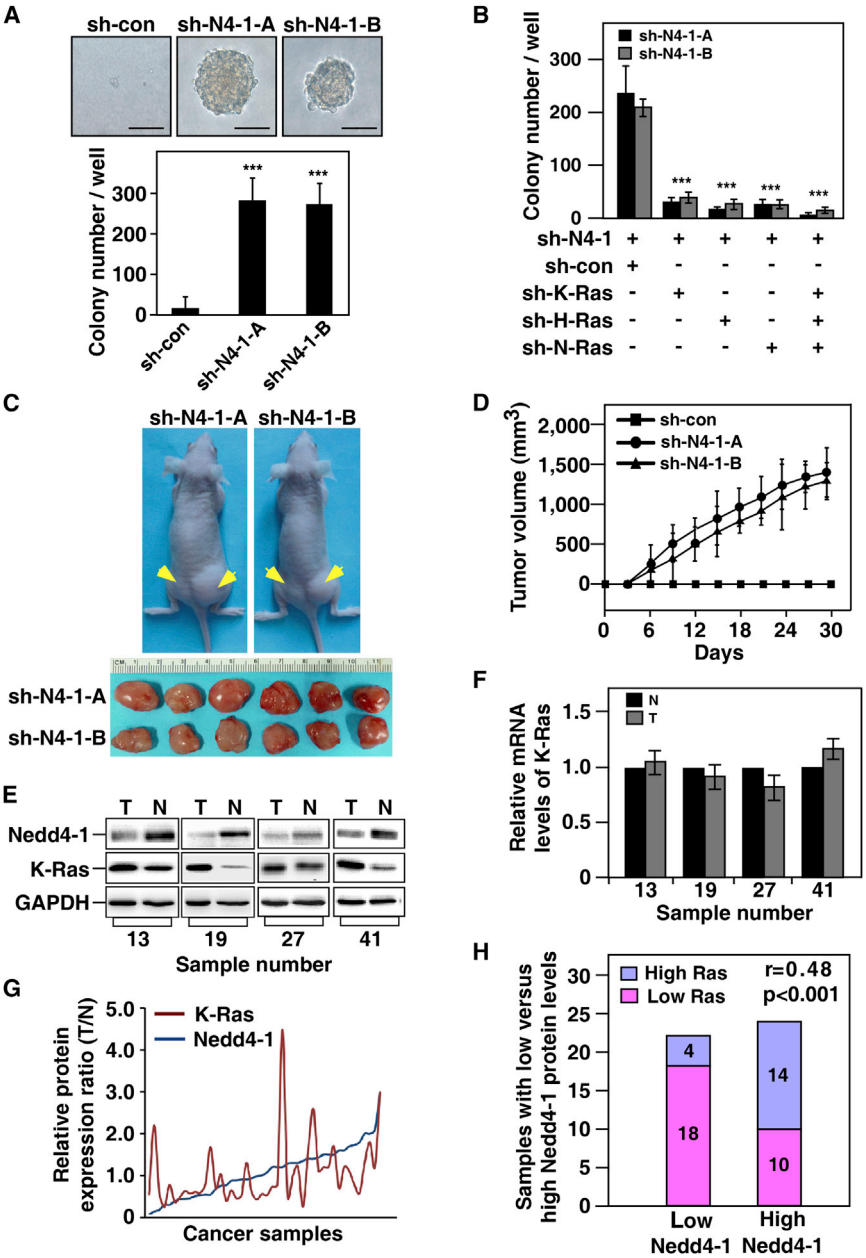

Figure 3. Nedd4-1 Suppresses Tumorigenesis by Targeting Ras

(A) Knockdown of Nedd4-1 induces transformation of NIH 3T3 cells. NIH 3T3 cells transduced with lentivirus encoding control shRNA or Nedd4-1 shRNA (sh-N4-1-A or sh-N4-1-B) were subjected to soft agar assay. The scale bars indicate 100 μm. Viable colonies formed were quantitated and plotted as mean ± SD of six independent experiments.

(B) Simultaneously silencing Ras inhibits Nedd4-1 knockdown-induced transformation of NIH 3T3 cells. NIH 3T3 cells transduced with indicated combinations of lentivirus encoding Nedd4-1 shRNA and Ras shRNA were applied to soft agar assay. Viable colonies formed were quantitated and plotted as mean ± SD of three independent experiments.

(C and D) Knockdown of Nedd4-1 induces tumorigenicity of NIH 3T3 cells in nude mice. Nude mice were subcutaneously injected with NIH 3T3 cells stably expressing control shRNA (left flank) or Nedd4-1 shRNA (right flank) to observe tumor development (C), and volumes of tumor were determined and plotted as mean ± SD of six independent experiments (D).

(E) Nedd4-1 and K-Ras present inverse correlations in some human colon cancer specimens. Protein levels of Nedd4-1 and K-Ras were determined by immunoblotting the protein extracts from tumor tissues (T) and their matched surrounding normal mucosal tissues (N). GAPDH was used as a loading control.

(F) Increase of K-Ras is not due to upregulation of its transcription. The mRNA levels of K-Ras in tumor (T) and matching normal tissues (N) were measured by real-time quantitative PCR using GAPDH as an internal control. Data are presented as mean ± SD of three independent experiments.

(G) The protein levels of Nedd4-1 and K-Ras in colorectal carcinomas. The protein levels of Nedd4-1 (blue line) and K-Ras (red line) observed in a series of 46 colorectal carcinomas were plotted in the order of increasing Nedd4-1 protein levels.

(H) Correlation between Nedd4-1 and K-Ras protein levels in colorectal cancer. A total of 46 samples of human colorectal cancer were classified into 2 groups based on the protein levels of Nedd4-1 in tumor tissues compared to surrounding normal tissues (low Nedd4-1, 22 samples; high Nedd4-1, 24 samples). The correlation coefficient r value was obtained by Spearman's rank correlation analysis.

two independent small hairpin RNAs (shRNAs) (sh-N4-1-A and sh-N4-1-B) targeting different regions of Nedd4-1 mRNA to knock down Nedd4-1 in NIH 3T3 cells, and then seeded the cells in soft agar. Strikingly, knockdown of Nedd4-1 dramatically induced colony formation of NIH 3T3 cells in soft agar (Figure 3A), revealing a role of Nedd4-1 in suppressing cell transformation. To assess whether Nedd4-1 knockdown-induced cell transformation is through upregulating Ras proteins, we knocked down both Ras and Nedd4-1. As shown in Figure 3B, simultaneous knockdown of Ras strongly suppressed Nedd4-1 knockdown-induced transformation of NIH 3T3 cells, confirming that the transformation induced by Nedd4-1 knockdown is dependent on Ras.

Next, we tested the in vivo tumor-forming capacity of NIH 3T3 cells with Nedd4-1 knocked down. To this end, we generated multiple NIH 3T3 clonal lines stably expressing sh-N4-1-A or sh-N4-1-B, and injected subcutaneously into athymic nude mice to inspect the tumor formation. Strikingly, both sh-N4-1-A and sh-N4-1-B-expressing cells formed large tumors within 1 month in nude mice, whereas NIH 3T3 cells with control shRNA did not (Figure 3C). The growth rates of the tumors were measured and plotted in Figure 3D. Thus, our results

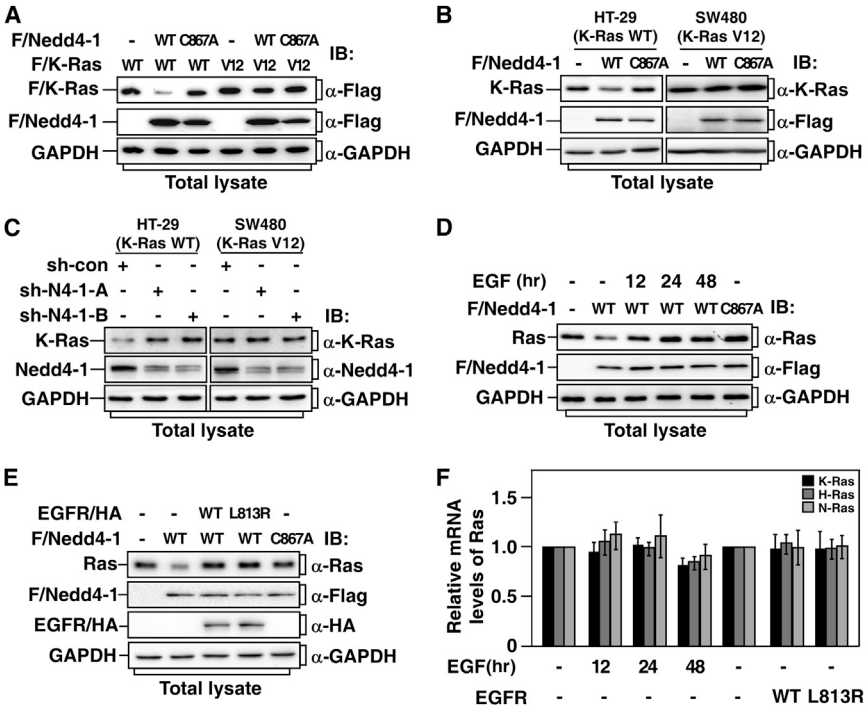

Figure 4. Ras Evades Nedd4-1-Mediated Degradation via Site Mutation or Consecutive Activation

(A) G12V mutation restrains Nedd4-1-mediated K-Ras degradation. HEK293T cells transfected with F/K-Ras (WT or V12) and F/Nedd4-1 (WT or C867A) as indicated were subjected to immunoblotting.

(B) Nedd4-1 overexpression decreases levels of WT K-Ras but not V12 mutant. Endogenous levels of WT or V12 K-Ras in HT-29 or SW480 cells overexpressing F/Nedd4-1 (WT or C867A) were examined by immunoblotting.

(C) Knocking down Nedd4-1 increases endogenous levels of WT K-Ras but not V12 mutant. HT-29 cells or SW480 cells were transduced with lentivirus encoding control shRNA or Nedd4-1 shRNA, and endogenous levels of WT K-Ras in HT-29 cells and V12 mutant in SW480 cells were examined by immunoblotting.

(D) EGF treatment inhibits Nedd4-1-mediated Ras degradation. HEK293T cells overexpressing F/Nedd4-1 (WT or C867A) were treated with or without EGF (50 ng/ml) for the indicated time and then subjected to immunoblotting.

(E) Overexpression of EGFR prevents Nedd4-1-mediated Ras degradation. Endogenous protein levels of total Ras in HEK293T cells transduced with indicated combinations of lentivirus encoding WT or constitutive active form (L813R) C-terminal HA-tagged EGFR (EGFR/HA) and F/Nedd4-1 (WT or C867A) were determined by immunoblotting the total cell lysates.

(F) EGF signaling-caused increase of Ras levels is not through upregulating Ras transcription. The mRNA levels of K-Ras, H-Ras, and N-Ras in HEK293T cells treated with or without EGF (50 ng/ml) for the indicated hours, or transfected with WT or L813R EGFR, were measured by real-time RT-PCR using GAPDH as an internal control. Data are presented as mean ± SD of three independent experiments.

clearly show that Nedd4-1 has a critical role in suppressing tumorigenesis.

To explore whether there is any correlation between Nedd4-1-mediated Ras degradation and human tumorigenesis, we analyzed specimens from 46 patients with colorectal cancer. We found that in four tumor samples, levels of Nedd4-1 proteins were decreased, whereas protein levels of K-Ras were increased in the tumor tissues compared with surrounding normal mucosal tissues (Figure 3E; Table S1), and the increase of K-Ras protein was not due to upregulation of K-Ras mRNA levels (Figure 3F). Intriguingly, the overall relationship between Nedd4-1 protein levels and K-Ras protein levels was not reversely correlated (Figure 3G), and in about 30% of tumor samples (14 out of 46), the levels of Nedd4-1 protein were upregulated and the Ras protein levels were not decreased (Figure 3H). We thus decided to pursue the underlying mechanism for this apparently conflicting phenomenon.

Oncogenic Activation of Ras Blocks Nedd4-1-Mediated Ras Degradation

Because oncogenic mutations of Ras occur frequently in human cancers (Pylayeva-Gupta et al., 2011), we wondered whether these mutations could inhibit Nedd4-1-mediated Ras degradation. To test this possibility, we examined the ability of Nedd4-1 to mediate degradation of various Ras mutants that commonly exist in cancer cells. Remarkably, many Ras mutations, especially on Gly12, significantly attenuated Nedd4-1-mediated Ras degradation (Figures 4A, S2A, and S2B). This was further confirmed by examining the endogenous levels of K-Ras G12V (V12) mutant that is harbored in the colon cancer cell line SW480. Overexpression of Nedd4-1 markedly decreased WT K-Ras in the colon cancer cell HT-29, whereas it had no effect on the steady-state levels of K-Ras V12 in SW480 (Figures 4B and S2C). Meanwhile, knockdown of Nedd4-1 increased levels of WT K-Ras protein in HT-29 but did not affect K-Ras V12 protein levels in SW480 (Figures 4C and S2D).

Ras small GTPases are physiologically activated by receptor tyrosine kinases such as EGFR, and overexpression of EGFR or dominant-active mutations of EGFR are frequently found in tumors (Downward, 2003). Therefore, we further tested whether EGF signaling could protect Ras proteins from Nedd4-1-mediated degradation. Indeed, treatment of EGF or overexpressing WT EGFR or dominant-active mutant EGFR L813R effectively rescued Ras from Nedd4-1-mediated degradation (Figures 4D, 4E, S2E, and S2F). Of note, treatment of EGF or overexpressing EGFRs did not upregulate mRNA levels of Ras (Figure 4F), indicating that EGF signaling is through inhibiting Nedd4-1-mediated Ras degradation to protect Ras.

Binding of GTP Attenuates Ubiquitination of Ras Mediated by Nedd4-1

To investigate the mechanism by which activation of Ras helps Ras proteins circumvent the control of Nedd4-1, we first

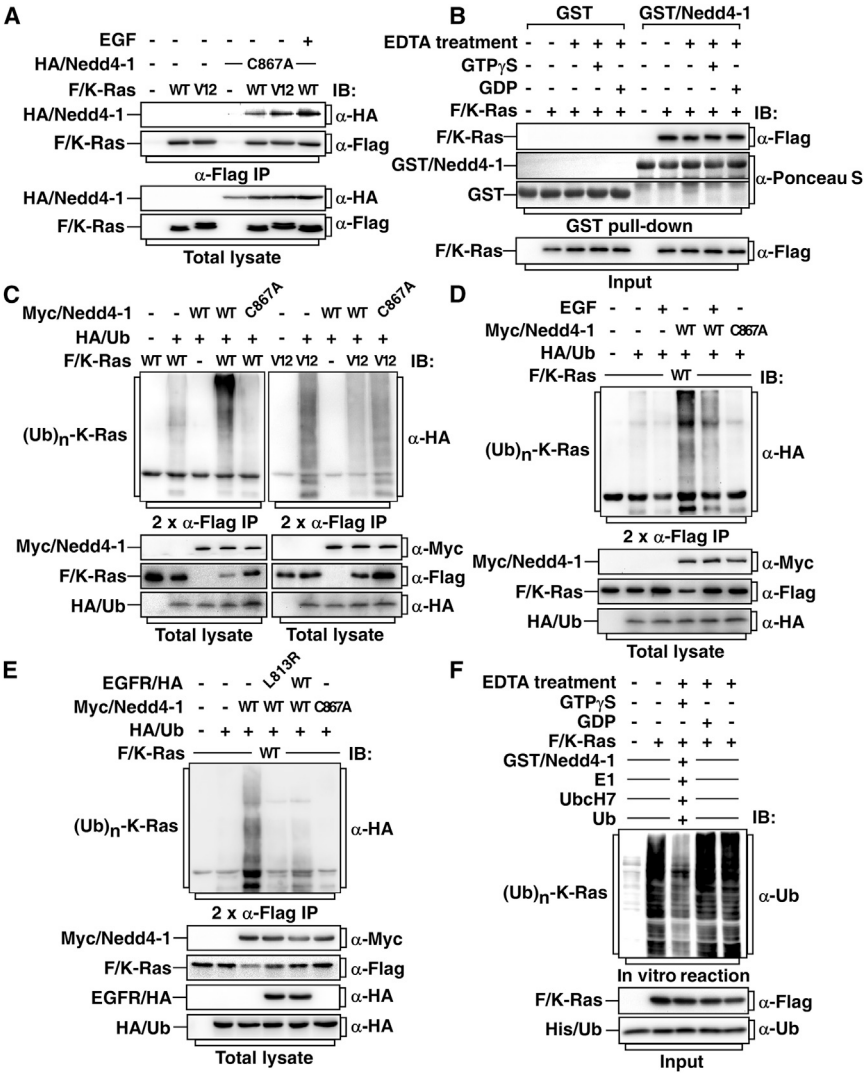

Figure 5. Oncogenic Mutation or Constant Activation Protects K-Ras by Attenuating Nedd4-1-Mediated Ubiquitination

(A) G12V mutation or EGF treatment does not inhibit binding of K-Ras to Nedd4-1. HEK293T cells transfected with F/K-Ras (WT or V12) and HA/Nedd4-1 C867A as indicated were treated with or without EGF (50 ng/ml for 24 hr) as indicated before being subjected to coimmunoprecipitation assay.

(B) Nedd4-1 interacts with both GDP- and GTPγS-bound forms of K-Ras in vitro. Nucleotide-free F/K-Ras obtained by EDTA treatment was loaded with GDP or GTPγS and then subjected to GST pull-down assay as indicated. GST and GST/Nedd4-1 were detected by Ponceau S staining.

(C) G12V mutation attenuates Nedd4-1-mediated K-Ras ubiquitination. HEK293T cells transfected with indicated combinations of F/K-Ras (WT or V12), Myc/Nedd4-1 (WT or C867A), and HA/Ub were subjected to ubiquitination assay.

(D) EGF treatment attenuates Nedd4-1-mediated K-Ras ubiquitination. HEK293T cells transfected with indicated combinations of F/K-Ras, Myc/Nedd4-1 (WT or C867A), and HA/Ub were treated with or without EGF (50 ng/ml) for 24 hr and then subjected to ubiquitination assay.

(E) Overexpression of EGFR diminishes Nedd4-1-mediated K-Ras ubiquitination. HEK293T cells transfected with indicated combinations of F/K-Ras, Myc/Nedd4-1 (WT or C867A), EGFR/HA (WT or L813R), and HA/Ub were subjected to ubiquitination assay.

(F) Load of GTPγS inhibits Nedd4-1-mediated K-Ras ubiquitination. Nucleotide-free F/K-Ras was loaded with GDP or GTPγS and then subjected to in vitro ubiquitination assay as indicated.

hypothesized that the interaction between Nedd4-1 and Ras might be disturbed by Ras mutation or EGF treatment. To our surprise, G12V mutation or EGF treatment did not block binding of K-Ras to Nedd4-1. As shown in Figure 5A, Nedd4-1 C867A displayed an equal binding ability to both WT and V12 K-Ras in cells, and GST/Nedd4-1 evenly interacted with GDP-bound, GTPγS-bound, and guanine-free forms of K-Ras in vitro (Figure 5B), suggesting that activation of Ras does not affect its binding affinity to Nedd4-1.

We then examined the effect of G12V mutation and EGF signaling on Nedd4-1-regulated ubiquitination of K-Ras. Indeed, the ubiquitination of K-Ras catalyzed by Nedd4-1 was significantly diminished by G12V mutation (Figure 5C) or EGF signaling (Figures 5D and 5E). Meanwhile, loading of GTPγS on K-Ras or G12V mutation markedly inhibited Nedd4-1-catalyzed K-Ras ubiquitin conjugation in vitro (Figures 5F and S3A). We further identified Lys5 as the ubiquitination site in K-Ras using mutational analysis (Figures S3B–S3D). Interestingly, the side chain of Lys5 goes through a significant conformational change when Ras undergoes interchanging between GDP- and GTP-bound states (Figures S3E–S3H), suggesting that binding of GTP might interrupt the access of ubiquitin to Lys5. Hence, our results indicated that aberrant activation of Ras, as a result of oncogenic mutations of Ras or persistent activation of EGFR, enables tumor cells to overcome Nedd4-1-mediated inhibition of Ras signaling by circumventing Nedd4-1-regulated Ras ubiquitination.

Ras Signaling Upregulates Transcription of Nedd4-1

Because we observed that in some tumor specimens both Nedd4-1 protein levels and K-Ras protein levels were upregulated (Figure 3H), we further examined the role of Ras signaling in regulation of Nedd4-1. Remarkably, overexpressing K-Ras (WT or V12) or treatment of EGF significantly increased endogenous levels of Nedd4-1 in HEK293T cells (Figures 6A, 6B, S4A, and S4B), suggesting that Ras signaling may upregulate Nedd4-1 levels. We examined the mRNA levels of Nedd4-1 in various types of cells by quantitative RT-PCR and found that overexpressing Ras or treatment of EGF markedly enhanced transcription of Nedd4-1 (Figures 6C and 6D), indicating a negative feedback regulatory loop between Nedd4-1 and Ras signaling.

We reinforced the role of Ras signaling in regulating Nedd4-1 transcription by analyzing published mRNA microarray data through the publicly available database Oncomine (http://www.oncomine.com). Based on the data of mRNA levels retrieved from a subset database containing expression profiles of 56 colorectal cancer samples (Figure S4C), we found that there were good positive correlations between K-Ras and Nedd4-1 ($r = 0.82$; $p < 0.001$) (Figures 6E and 6F) and between EGFR and Nedd4-1 ($r = 0.84$; $p < 0.001$) (Figures 6G and 6H). Moreover, we detected activated EGFR levels using a phosphotyrosine-specific antibody (anti-pY1068-EGFR) and confirmed that the protein levels of Nedd4-1 were increased when levels of phosphorylated EGFR were upregulated in tumor samples by western blot (Figure 6I; Table S1) and immunohistochemistry assays (Figure 6J).

Ras Oversignaling Promotes PTEN Degradation via Nedd4-1

Given the fact that Nedd4-1 levels were upregulated in a large percentage of tumor samples (Figure 3H), which suggests that cancer cells may take advantage of Nedd4-1 to promote tumor progression when Ras proteins overcome the control of Nedd4-1, we next decided to explore the underlying role of Ras signaling-enhanced transcription of Nedd4-1 in cancer cells. Because a previous study reported that Nedd4-1 could target tumor suppressor PTEN for degradation (Wang et al., 2007), which plays an important role in promoting tumor development, we therefore examined whether Ras activation had any effect on protein levels of PTEN. Indeed, while the Nedd4-1 levels were increased upon K-Ras overexpression or EGF treatment, the PTEN levels were significantly decreased in various types of cancer cells including HeLa, HT-29, BGC-823, and HepG2 (Figures 7A, 7B, and S5A–S5F), suggesting that Ras signaling may promote PTEN degradation through upregulating Nedd4-1 levels in cancer cells. Moreover, expression of exogenous Nedd4-1 decreased endogenous PTEN levels (Figures 7C and S5G), whereas knockdown of Nedd4-1 resulted in an increase of PTEN levels (Figures 7D and S5H). Consistently, knockdown of Nedd4-1 in SW480 upregulated the PTEN levels but did not affect the endogenous K-Ras V12 levels (Figures S5I and S5J), and significantly impaired the colony formation of SW480 in soft agar (Figure S5K).

To confirm that the decrease of PTEN caused by hyperactive Ras signaling is through Nedd4-1, we first examined whether Ras activation could affect transcription of PTEN. As shown in Figure 7E, the mRNA levels of PTEN were not significantly changed in response to Ras overexpression or EGF treatment, indicating that Ras signaling-induced decrease of PTEN was not due to downregulating PTEN transcription. We next knocked down Nedd4-1 and tested whether PTEN levels could still be decreased by activation of Ras. Indeed, Ras overexpression or EGF treatment failed to downregulate PTEN when Nedd4-1 was knocked down, confirming that the decrease of PTEN by Ras signaling was dependent on Nedd4-1 (Figures 7F, 7G, S5L, and S5M).

To validate the correlation between Ras oversignaling and PTEN levels in human cancers, we examined the PTEN levels in human colorectal cancer samples. Significantly, PTEN levels were decreased in about 80% (12 out of 14) of the tumor samples with high Ras and Nedd4-1 levels (Figure 7H; Table S1), and the overall relationship between protein levels of Ras and PTEN in the 46 colorectal tumor samples was reversely correlated ($r = -0.62$) (Figure 7I), suggesting a malignant role of Ras oversignaling in downregulating PTEN through stimulating Nedd4-1 expression. Furthermore, we confirmed that Ras-induced downregulation of PTEN was not through affecting PTEN mRNA levels, by analyzing mRNA microarray data through Oncomine (Figures S5N and S5O). Thus, our study demonstrated a fine-tuned interplay between Nedd4-1, Ras and PTEN. Constitutive activation of Ras by oncogenic mutations or growth factors impedes Nedd4-1-mediated Ras degradation, in turn causes Nedd4-1 overly expressed to target PTEN for degradation, thereby switching Nedd4-1 to an oncoprotein, leading to tumor progression.

DISCUSSION

Mechanistic studies for controlling Ras activity have been mainly focused on the regulation of cycling between GDP-bound and GTP-bound forms of Ras proteins regulated by GEFs and GAPs, and the membrane association mediated by posttranslational modification such as farnesylation and palmitoylation at the carboxy-terminal CAAX motif (Ahearn et al., 2012). Emerging evidence suggested that ubiquitination of Ras plays important roles in regulating Ras stability and activity as well (Pfleger, 2011). Recent studies showed that mono- or diubiquitination restricted activities of H-Ras and N-Ras (Colicelli, 2010; Xu et al., 2010; Yan et al., 2009, 2010), whereas mono-ubiquitination of K-Ras promoted its activity (Sasaki et al., 2011). It has been reported that H-Ras can also be targeted for polyubiquitination and degradation by β-TrCP (Kim et al., 2009), and aberrant activation of Wnt/β-catenin signaling promotes intestinal tumorigenesis by stabilizing H-Ras (Jeong et al., 2012). However, it remains unclear whether it has to be distinct ubiquitin ligases for different Ras proteins, or one same ubiquitin ligase can target all three forms of Ras, and what roles of Ras ubiquitination would exert in tumorigenesis (Pfleger, 2011).

Our current study demonstrates Nedd4-1 as a general E3 ubiquitin ligase for controlling the abundance of all the three major forms of Ras, revealing a fundamental role of Nedd4-1 in regulating Ras signaling. Importantly, the interplay between Ras signaling-regulated transcription of Nedd4-1 and Nedd4-1-mediated Ras degradation comprises a negative feedback regulatory loop. We found that a near-complete knockdown of Nedd4-1 caused cell senescence and that raising Ras to a moderately increased level by restrained knockdown of Nedd4-1 promotes cell growth and transformation. These observations are in good agreement with previous reports that low levels activation of Ras promoted cell proliferation, whereas high levels activation of Ras caused oncogene-induced senescence (OIS) (DeNicola and Tuveson, 2009; Sarkisian et al., 2007; Serrano et al., 1997). Hence, the feedback regulatory loop between Ras and Nedd4-1 is critical for finely adjusting Ras signaling, which might be essential for maintaining homeostasis of cell growth and senescence. Interruption of this regulatory loop may result in dysregulation of Ras signaling and tumorigenesis.

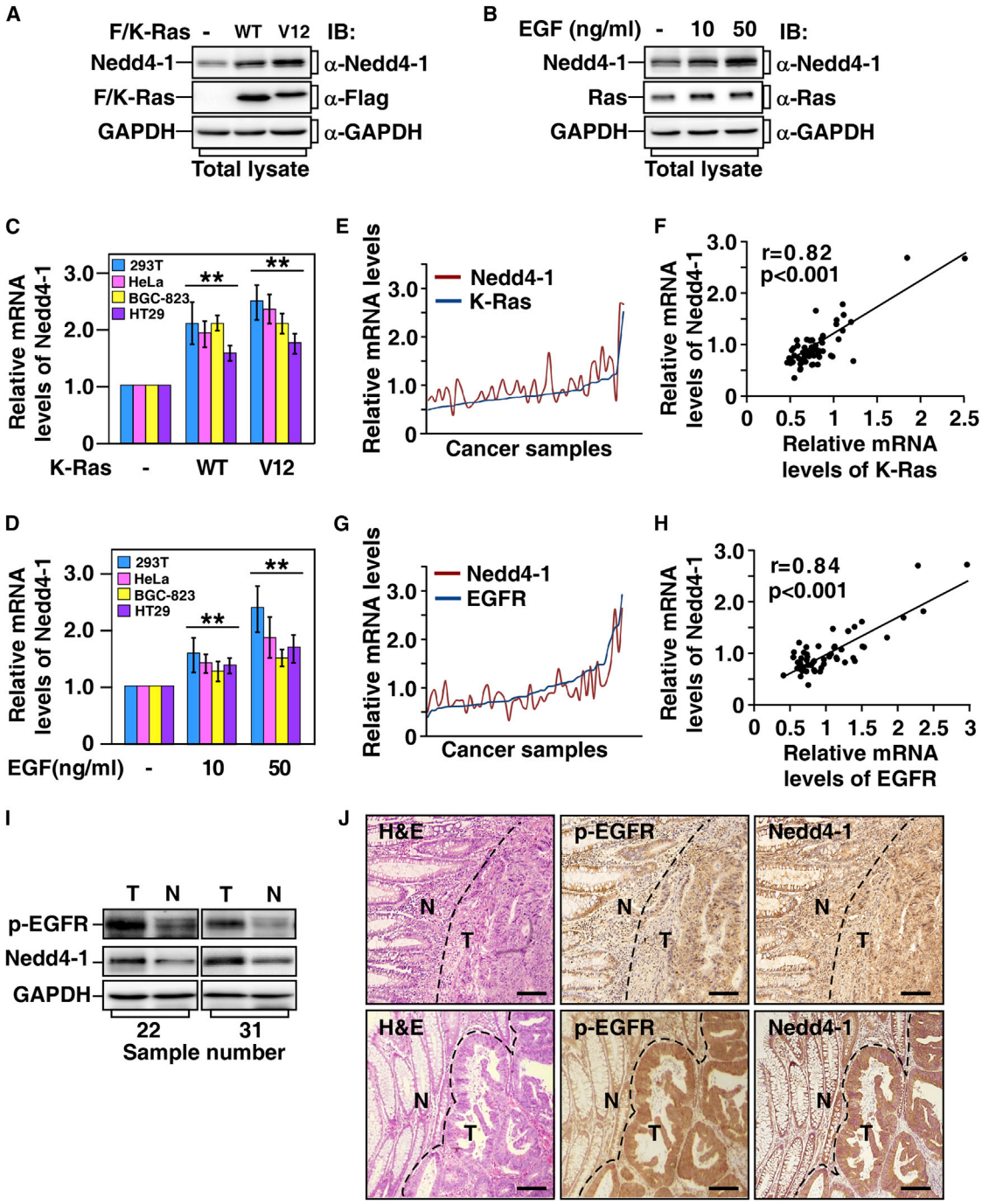

Figure 6. Ras Activation Upregulates Nedd4-1 Transcription

(A) Overexpressing Ras increases endogenous Nedd4-1 levels. Nedd4-1 levels in HEK293T cells transfected with F/K-Ras (WT or V12) were examined by immunoblotting.

(B) Treatment of EGF increases endogenous levels of Nedd4-1. HEK293T cells were treated with different doses of EGF as indicated for 36 hr before being applied to western blot to determine the endogenous levels of Nedd4-1.

(C) Ras overexpression enhances Nedd4-1 transcription. The mRNA levels of Nedd4-1 in HEK293T, HeLa, BGC-823, or HT-29 cells transfected with K-Ras (WT or V12) were measured by real-time RT-PCR using GAPDH as an internal control. Data are presented as mean ± SD of three independent experiments.

(D) EGF treatment upregulates Nedd4-1 transcription. The mRNA levels of Nedd4-1 in HEK293T, HeLa, BGC-823, or HT-29 cells treated 2 hr with different doses of EGF as indicated were measured by real-time quantitative PCR using GAPDH as an internal control. Data are presented as mean ± SD of three independent experiments.

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We have also found that activated Ras proteins, as a result of either activating mutations or EGF signaling, are resistant to Nedd4-1-mediated degradation. Aberrant activation of Ras is a common prelude to tumorigenesis, which is associated with about 30% of all human cancers. Constitutively active Ras mutants constantly activate downstream signaling pathways, thereby acting as a major etiological factor for cancer development. Apparently, our study provides a fundamental mechanism by which oncogenic activation of Ras is such a prevalent cause for tumor development. It would be necessary for normal cells to temporarily relieve Nedd4-1-mediated Ras degradation in response to upstream growth signaling such as EGF; however, activating mutations of Ras or persistent stimulation of EGF signaling will utterly block Nedd4-1-mediated Ras degradation, therefore enabling Ras proteins to escape the control of Nedd4-1, which might be essential for Ras-driven tumorigenesis. Moreover, what is worse is that the uncontrolled Ras signaling noxiously upregulates Nedd4-1 levels to enhance Nedd4-1-mediated PTEN degradation in cancer cells, thus promoting malignancy of cancer cells. It is worthwhile to point out that the Nedd4-1-mediated PTEN degradation might primarily happen in cancer cells under certain oncogenic circumstances because previous studies showed that Nedd4-1 was not responsible for regulation of PTEN in primary cells (Cao et al., 2008; Fouladkou et al., 2008), and tyrosine kinase Rak acted as a tumor suppressor by inhibiting Nedd4-1-regulated PTEN degradation (Yim et al., 2009).

It is widely accepted that cancer cells can take advantage of genes or signaling pathways that are needed for the autonomy of cellular proliferation involved in normal growth or tissue renewal (Condeelis et al., 2005). We present here another example for this concept that cancer cells hijack the E3 ubiquitin ligase Nedd4-1, switch it to a proto-oncoprotein, and thereby promote the tumor progression. Hence, we have to reshape the strategy for cancer therapy using Nedd4-1 as a molecular target. It would be necessary to further dissect the mechanisms for the two opposite roles of Nedd4-1 in tumorigenesis in order to specifically block Nedd4-1-mediated PTEN degradation while retaining its activity toward Ras proteins.

EXPERIMENTAL PROCEDURES

Antibodies and Chemical Reagents

The mouse anti-K-Ras monoclonal (ab16795), rabbit anti-H-Ras monoclonal (ab16881), goat anti-N-Ras polyclonal (ab77392), and mouse anti-Ras (for total Ras proteins) monoclonal (ab55391) antibodies were from Abcam; rabbit anti-Nedd4-1 polyclonal antibody (07-049) was from Millipore; mouse anti-FLAG M2 antibody (F1804) was from Sigma-Aldrich; rat anti-HA monoclonal antibody (11867431001) was from Roche; mouse anti-Myc (SC-40), anti-Ub (SC-8017), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; SC-61) monoclonal antibodies were from Santa Cruz Biotechnology; and rabbit anti-pEGFR (Tyr1068) (phospho-EGFR) (3777) and rabbit anti-PTEN monoclonal (9188) antibodies were from Cell Signaling Technology. The chemical reagents chloroquine, cycloheximide, EGF, GTPγS, and GDP were from Sigma-Aldrich; MG-132 was from Boston Biochem.

DNA Constructs

Human Nedd4-1 and Nedd4-2 were generous gifts from Dr. W. Sundquist, and human EGFR was a kind gift from Dr. J. Han. Different tagged Nedd4-1 and FLAG-Nedd4-2 were generated by subcloning PCR products of Nedd4-1 and Nedd4-2 into pCMV5 vector. The catalytic inactive mutants of Nedd4-1 C867A and Nedd4-2 C942A and constitutive active mutant EGFR-L813R were generated by PCR-based site-directed mutagenesis according to previous reports by Chung et al. (2008), Hamilton et al. (2001), and Paez et al. (2004). K-Ras, H-Ras, and N-Ras were cloned from HEK293T cells using RT-PCR, and various mutations of Ras were introduced by PCR-based site-directed mutagenesis. Nedd4-1 and Ras were subcloned into pGEX4T-1 vector for bacterial expression. For gene silencing, shRNAs were all generated by using pLL3.7 vector (Addgene; Table S2).

Cell Culture, Transfection, Lentivirus Infection, and Stable Cell Line Generation

Mouse fibroblast NIH 3T3, MEF, HEK293T, human cervical cancer HeLa, and human hepatocellular carcinoma HepG2 cells were grown in high-glucose Dulbecco's modified Eagle's medium (Invitrogen), human colon cancer SW480 cells and human gastric cancer BGC-823 cells were grown in RPMI-1640 medium (Invitrogen), and human colon cancer HT-29 cells were grown in McCoy's 5A medium (Sigma-Aldrich), all supplemented with 10% (v/v) fetal bovine serum (Gibco) and 100 U/ml streptomycin and penicillin (Millipore), at 37°C in a humidified 5% CO_2 incubator. HEK293T, NIH 3T3, SW480, HepG2, and HeLa cells were obtained from ATCC. HT-29 and BGC-823 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai). MEF cells prepared from embryos of mice and immortalized using large T antigen were a kind gift from Dr. J Han.

HEK293T cells were transiently transfected using the calcium phosphate methods as previously described (Wang et al., 2006). HeLa, SW480, HepG2, and BGC-823 cells were transfected with Lipofectamin 2000 (Invitrogen) or electroporation (Bio-Rad). Recombinant lentivirus used to infect NIH 3T3, MEF, and HT-29 cells for shRNA or protein expression was generated by the ViraPower Lentiviral Expression System (Invitrogen) according to the manufacturer's instruction.

To generate Nedd4-1 shRNA stably expressing cells, NIH 3T3 cells were transduced with lentivirus encoding neomycin-resistance gene and control shRNA or Nedd4-1 shRNA. Neomycin-resistant colonies were then screened by immunoblotting to examine Nedd4-1 expression level with anti-Nedd4-1 antibody.

(E) The mRNA levels of K-Ras and Nedd4-1 in colorectal carcinomas. The mRNA levels of K-Ras (blue line) and Nedd4-1 (red line) observed in a series of 56 colorectal carcinomas were plotted in the order of increasing K-Ras expression. The mRNA data were obtained from the Oncomine database (http://www.oncomine.com).

(F) Positive correlation between mRNA levels of K-Ras and Nedd4-1. The correlation between Nedd4-1 and K-Ras mRNA expression in 56 human colon cancer tissues was analyzed by Pearson's correlation analysis with a coefficient of 0.82 ($p < 0.001$).

(G) The mRNA levels of EGFR and Nedd4-1 in colorectal carcinomas. Same as in (E), except that mRNA levels of EGFR instead of K-Ras were used.

(H) Positive correlation between mRNA levels of EGFR and Nedd4-1. Same as in (F), except that mRNA levels of EGFR instead of K-Ras were used. Pearson's correlation coefficient (r) was 0.84 ($p < 0.001$).

(I) Positive correlation between protein levels of Nedd4-1 and phospho-EGFR in colorectal tumor samples. The protein levels of Nedd4-1 and phospho-EGFR (pY1068) were determined by immunoblotting the protein extracts from tumor tissues (T) and their matched surrounding normal mucosal tissues (N). GAPDH was used as a loading control.

(J) Positive correlation between expression of Nedd4-1 and phospho-EGFR in colorectal cancer. The expression of Nedd4-1 in tumor tissue (T) and surrounding normal mucosal tissue (N) in typical colorectal tumor samples with (bottom) or without (top) upregulation of phospho-EGFR (pY1068) was detected by immunohistochemistry assay. The scale bars indicate 200 μm.

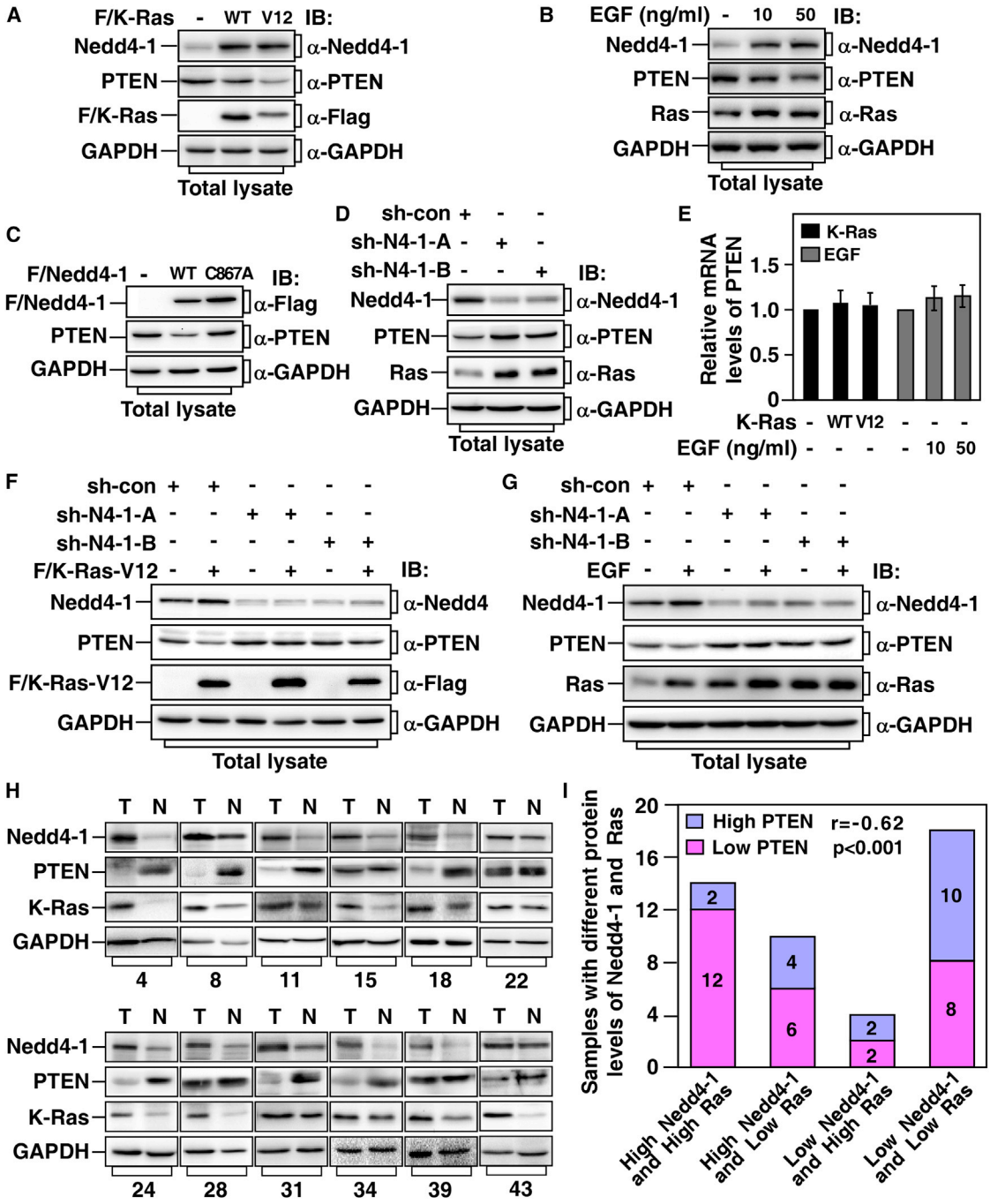

Figure 7. Ras Oversignaling Promotes PTEN Degradation through Upregulating Nedd4-1

(A) Overexpression of K-Ras decreases PTEN levels. Endogenous levels of Nedd4-1 and PTEN in HeLa cells overexpressing F/K-Ras (WT or V12) were examined by immunoblotting.

(B) EGF treatment decreases PTEN levels in HeLa cells. HeLa cells were treated overnight with or without indicated doses of EGF and then subjected to western blot.

(C) Overexpressing Nedd4-1 decreases PTEN levels. Endogenous PTEN levels in HeLa cells overexpressing F/Nedd4-1 (WT or C867A) were determined by immunoblotting.

(D) Knocking down Nedd4-1 increases PTEN levels. HeLa cells expressing control or Nedd4-1 shRNA were subjected to immunoblotting to determine the protein levels.

(E) Activation of Ras signaling does not affect PTEN transcription. The mRNA levels of PTEN in HeLa cells transduced with lentivirus encoding K-Ras (WT or V12) or treated with indicated doses of EGF were measured by real-time quantitative PCR using GAPDH as an internal control. Data are presented as mean ± SD of three independent experiments.

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Immunoprecipitation, Immunoblotting, GST Pull-Down, and Ubiquitination Assays

Immunoprecipitation (IP), immunoblotting, GST pull-down, and ubiquitination assays were performed as previously described (Wang et al., 2006). Briefly, cell lysates prepared using ice-cold lysis buffer TNTE 0.5% (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100, containing 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM PMSF) were applied to IP or immunoblotting assays with appropriate antibodies. For ubiquitination assay in cells, cell lysates were subjected to anti-FLAG IP, eluted by 5 min boiling in 1% SDS, then diluted ten times in lysis buffer TNTE 0.5% and reimmunoprecipitated with anti-FLAG (2× IP). The ubiquitin-conjugated proteins were detected by immunoblotting. For GST pull-down and in vitro ubiquitination assays, bacterially expressed GST/Nedd4-1 (WT or C867A) was purified using glutathione Sepharose beads in TNTE 0.5% buffer. GST/FLAG/Ras fusion proteins were expressed in *E. coli* and purified in the presence of Mg^{2+} (Hall and Self, 1986). Nonfused FLAG/Ras proteins were obtained by tobacco etch virus protease cleavage, and nucleotide-free state and a GDP- or GTPγS-bound form of FLAG/K-Ras were generated as previously described (Wang et al., 2006).

Affinity Purification of Nedd4-1 Binding Proteins

Halo-tagged Nedd4-1 C867A transiently expressed in HEK293T cells was purified with Magne HaloTag Beads (Promega) in Halo-tag protein purification buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM dithiothreitol, 0.05% NP-40, and 1 mM phenylmethanesulfonylfluoride) according to the manufacturer's instruction. The bead-bound proteins were eluted by boiling 3 min with loading buffer and then subjected to SDS-PAGE. The proteins were detected with silver staining, and specific bands were excised and analyzed by mass spectrometry.

Immunofluorescence and Immunohistochemistry Assays

HeLa cells transiently transfected with HA-tagged Nedd4-1 C867A and FLAG-tagged K-Ras, H-Ras, or N-Ras were grown on glass coverslips. Cells were washed in PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.25% Triton X-100. FLAG-tagged Ras proteins were detected using mouse anti-FLAG M2 antibody followed by Alexa Fluor 555-conjugated donkey anti-mouse secondary antibody (Invitrogen), and HA-tagged Nedd4-1 was stained using rat anti-HA antibody followed by Alexa Fluor 488-conjugated donkey antibody to rat immunoglobulin G (Invitrogen). Images were acquired by using a Zeiss LSM 780 laser-scanning confocal microscope and ZEN 2010 software (Carl Zeiss).

For immunohistochemistry assay, paraffin-embedded tissue samples were sectioned, deparaffinized, and rehydrated. Sections were stained with hematoxylin and eosin (H&E) to examine the morphology. For immunohistochemical staining, the sections were boiled 20 min in sodium citrate/citric acid mixture (pH 6.0) for antigen retrieval and then pretreated with peroxidase blocking buffer (Maxim) for 20 min at room temperature. After being blocked with 5% normal goat serum for 1 hr at room temperature, the slides were incubated with appropriate primary antibodies overnight at 4°C. The UltraSensitive SP kit (Maxim) was then used to detect the specific primary antibodies.

Cell Transformation Assay

Forty-eight hours after infection, NIH 3T3 or SW480 cells transduced with lentivirus encoding control shRNA or Nedd4-1 shRNA were applied to soft agar assay to determine the transformation ability. The cells were harvested and seeded at 1×10^4 per 35 mm culture dish in 1.5 ml of 0.35% (w/v) Noble agar (Becton Dickinson) with complete medium. The dishes were precoated with 1.5 ml of 0.6% (w/v) Noble agar, and 0.5 ml of overlay medium was added after cell plating. The cells were cultured at 37°C in a humidified 5% CO_2 incubator for 1–3 weeks. The overlay medium was changed every 3 days. Colonies formed in soft agar were photographed using a Nikon eclipse Ti-U inverted microscope.

Tumorigenicity Assay in Nude Mice

NIH 3T3 cells stably expressing control shRNA or Nedd4-1 shRNA were used for the tumorigenicity assays. The cells were harvested at logarithmic growth phase by trypsinization, washing, and resuspending in PBS. The cells ($0.5–1 \times 10^7$ cells in 150 μl PBS) were subcutaneously injected in nude mice, and the growth of tumors was monitored for 1 month. For each shRNA, six independent clonal lines of NIH 3T3 cells were used for the injection. All animals were housed and handled with protocols approved by Institutional Animal Care and Use Committee.

Patient Samples

Primary human colorectal tumor samples and corresponding adjacent normal tissues were obtained in accordance with research ethics board approval from Xiamen University and Zhongshan Hospital. Informed consent was obtained from all patients. Briefly, all the samples taken after surgery were stocked in liquid nitrogen for further RNA extraction or immunoblotting assay, or embedded with paraffin for further immunohistochemistry assay.

Quantitative RT-PCR

Total RNAs were extracted from tissues or cells using TRIzol (Invitrogen), and cDNA was synthesized with ReverTra Ace qPCR RT kit (Toyobo). Real-time quantitative PCR was performed using SYBR Green PCR Mix (Roche) according to the manufacturer's protocol on a Mastercycler EP gradient S RealPlex^2 (Eppendorf). The relative changes of gene expression were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to *GAPDH* (Table S3).

Statistical Analysis

Data were expressed as mean ± SD of at least triplicate experiments. For western blot, protein bands were visualized by a Bio-Rad Molecular Imager ChemiDoc XRS system and analyzed with Image Lab software (Bio-Rad). The data significance was evaluated using Student's t test. $p < 0.05$ was considered a statistically significant difference (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.045.

AUTHOR CONTRIBUTIONS

T.Z., Q.W., and J.F. designed and carried out experiments. Q.L., J.B., W.D., Y.Q., S.Z., W.Z., H.L., and M.W. performed experiments. B.L. contributed materials and wrote the manuscript. X.D. and D.Z. contributed materials. Z.Y. designed experiments and interpreted data. H.-R.W. conceived and designed experiments, interpreted data, and wrote the manuscript.

(F) Ras overexpression-induced PTEN degradation is dependent on Nedd4-1. HeLa cells expressing indicated combinations of F/K-Ras V12 and control or Nedd4-1 shRNA were subjected to immunoblotting to examine the endogenous levels of Nedd4-1 and PTEN.

(G) EGF treatment-induced decrease of PTEN is dependent on Nedd4-1. HeLa cells transduced with lentivirus encoding control shRNA or Nedd4-1 shRNAs were treated overnight with or without EGF (50 ng/ml) as indicated. The endogenous Nedd4-1, PTEN, and Ras levels were determined by western blot.

(H) K-Ras and PTEN are reversely correlated in human colorectal cancer samples with high levels of Ras and Nedd4-1. Protein levels of PTEN, Nedd4-1, and K-Ras were determined by immunoblotting the protein extracts from tumor tissues (T) and their matched surrounding normal mucosal tissues (N). GAPDH was used as a loading control.

(I) Correlation between protein levels of K-Ras and PTEN. A total of 46 samples of human colorectal cancer were classified into 4 groups based on the protein levels of Nedd4-1 and K-Ras in tumor tissues compared to surrounding normal tissues. Indicated correlation coefficient r value was calculated by Spearman's rank correlation analysis.

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