Oncogenesis driven by the Ras/Raf pathway requires the SUMO E2 ligase Ubc9

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The small GTPase KRAS is frequently mutated in human cancer and currently there are no targeted therapies for KRAS mutant tumors. Here, we show that the small ubiquitin-like modifier (SUMO) pathway is required for KRAS-driven transformation. RNAi depletion of the SUMO E2 ligase Ubc9 suppresses 3D growth of KRAS mutant colorectal cancer cells in vitro and attenuates tumor growth in vivo. In KRAS mutant cells, a subset of proteins exhibit elevated levels of SUMOylation. Among these proteins, KAP1, CHD1, and EIF3L collectively support anchorage-independent growth, and the SUMOylation of KAP1 is necessary for its activity in this context. Thus, the SUMO pathway critically contributes to the transformed phenotype of KRAS mutant cells and Ubc9 presents a potential target for the treatment of KRAS mutant colorectal cancer.

KRAS | SUMO | Ubc9 | transformation | colorectal cancer

The Ras family of small GTPases are signal transduction molecules downstream of growth factor receptors. Ras activates a number of downstream effector pathways to regulate cell proliferation, survival and motility, these effectors include the MAP kinase (MAPK) pathway, the PI3-kinase (PI3K) pathway, the small GTPases RalA, RalB, and Rho, and phospholipase-Ce (1). Activating mutations in Ras are frequently found in human malignancies, with mutations in the *KRAS* gene being particularly prevalent. *KRAS* mutations occur in ~60% of pancreatic ductal carcinomas, 26% of lung adenocarcinomas, and 45% of colorectal carcinomas, as well as a significant fraction of ovarian, endometrial, and biliary track cancers (2, 3). A salient hallmark of the Ras oncogene is its ability to transform cells to enable anchorage-independent 3D colony growth in vitro and tumor growth in vivo. Consequently, Ras mutant cancer cells often exhibit oncogene addiction to Ras such that extinction of the Ras oncogene leads to either a reversion of the transformed phenotype or loss of viability (4, 5). Therapeutically, the Ras oncoprotein has proven pharmacologically intractable thus far: intensive drug screening efforts have not yielded high-affinity, selective Ras inhibitors. Farnesyltransferase inhibitors that aimed to block Ras membrane localization are ineffective against KRAS because of its alternative geranylgeranylation. Inhibitors targeting Ras effector kinases, including MEK, PI3K, and Akt, are currently undergoing clinical evaluations, but they have yet to demonstrate clear clinical benefits (6). Thus, KRAS mutant tumors represent a class of “recalcitrant cancer” with urgent, unmet therapeutic needs.

To gain new insight into the genetic dependencies of Ras mutant cancers and discover new therapeutic targets, we and others have previously carried out genome-wide synthetic lethal screens in KRAS mutant and WT cells to identify genes whose depletion leads to greater toxicity in KRAS mutant cells. In our screen we found a wide array of genes, many of which are involved in cellular stress response, that are required to maintain the viability of KRAS mutant cells (7). We proposed the concept of “nononcogene addiction” to explain the heightened dependency

of cancer cells on stress response and other indirect cellular pathways for survival, and we suggested that this form of addiction could be exploited for therapeutic gain (8).

In our primary screen we identified the small ubiquitin-like modifier (SUMO) E2 ligase Ubc9 (encoded by the *UBE2I* gene) and the E1 ligase subunit SAE1 as candidate KRAS synthetic lethal partners. Similar to the ubiquitin pathway, the SUMO pathway modulates the function and stability of cellular proteins through the reversible conjugation of SUMO on their lysine residues, often in a “ΨKxE” motif (9). In human, the SUMO pathway consists of three SUMO proteins (SUMO1, SUMO2, and SUMO3), a single heterodimeric E1 ligase SAE1/UBA2, a single E2 ligase Ubc9, and several E3 ligases. SUMO proteins are conjugated onto target proteins either directly by Ubc9 or through a family of E3s, and removed by the sentrin/SUMO-specific protease (SENP) family of SUMO peptidases. SUMOylation occurs in a highly dynamic manner in the cell and substrate proteins can be modified with either mono- or poly-SUMOylation. The SUMO pathway plays a critical role in cellular stress response, such as DNA damage, genomic stability, and heat shock (10–12), and it has also been recently implicated in prostate and breast cancer (13–16). However, the role of this pathway in KRAS mutant cancers is not clear.

In this study we provide evidence for the requirement for the SUMO pathway in the transformation growth of KRAS mutant colorectal cancer (CRC) cells. We found that these cells are highly dependent on Ubc9 for their clonogenic growth under both anchorage-dependent (AD) and anchorage-independent (AI) conditions. Quantitative proteomics analysis revealed that

Significance

Currently there are no targeted therapies for KRAS mutant cancer. Our study uncovers a critical role of the small ubiquitin-like modifier (SUMO) E2 ligase Ubc9 in sustaining the transformation growth of KRAS mutant colorectal cancer cells, thus establishing a functional link between the SUMO pathway and the *KRAS* oncogene. SUMO ligases are poorly explored drug targets; our work suggests that targeting the SUMO pathway, and Ubc9 in particular, could be potentially useful for the treatment of KRAS mutant colorectal cancers.

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Fig. 1. The SUMO pathway sustains clonogenic growth in KRAS mutant cells. (A) SAE1 and Ubc9 depletion modestly impair the viability of KRAS mutant DLD-1 and HCT116 cells. Viability of isogenic KRAS mutant and WT cell lines were assessed 5 d after shRNA transduction ($P < 0.05$). (B) Ubc9 knockdown significantly inhibits 2D clonogenic growth of KRAS mutant cells as measured by AD colony assay ($P < 0.05$). (C) Ubc9 and SAE1 knockdown significantly decreases AI colony numbers of KRAS mutant DLD-1 and HCT116 cells in 3D soft-agarose assay ($P < 0.05$ compared with shCtrl). (D) The E2 ligase activity of Ubc9 is required for AD (Left) and AI (Right) colony growth of KRAS mutant DLD-1 cells, as the phenotype of shUbc9#4 could be rescued by the expression of shRNA-resistant WT Ubc9 cDNA but not by the catalytically inactive Ubc9 mutant ($P < 0.05$). Error bars represent SEM of three independent experiments.

the SUMOylation patterns of a subset of cellular proteins are altered by the *KRAS* oncogene, and these SUMO target proteins functionally support the 3D growth of KRAS mutant cells. Our findings thus provide evidence that the SUMO pathway is critical for the transformation growth of KRAS mutant cancer cells, and suggests Ubc9 could be a potential drug target.

Results

KRAS-Driven Transformation Requires SUMO Ligases. The SUMO E1 ligase gene *SAE1* and the E2 ligase gene *UBE2I/Ubc9* scored as candidate KRAS synthetic lethal partners in our genome-wide shRNA screen (7). Although both scored moderately in the primary screen, they attracted our attention because they constitute the sole E1 and E2 SUMO ligase, respectively, and thus would critically control the activity of this pathway. We validated several shRNAs that effectively depleted SAE1 and Ubc9 and, in turn, reduced global protein SUMOylation (Fig. S1A and B). We next tested the effect of these shRNAs on the viability and clonogenic growth of isogenic KRAS mutant and WT DLD-1 and HCT116 CRC cell lines (4). Under normal 2D culture conditions, depletion of SAE1 and Ubc9 had a moderate impact on the growth of KRAS DLD-1 mutant cells that is associated with elevated levels of apoptosis in these cells (Fig. 1A and Fig. S1C). In 2D clonogenic assay, however, Ubc9 depletion strongly and selectively suppressed AD colony growth of KRAS mutant cells (Fig. 1B). In this KRAS isogenic system, the KRAS mutant cells but not KRAS WT cells are transformed and can form AI colonies under 3D growth conditions in soft agarose (4). Depletion of Ubc9 and SAE1 also strongly inhibited AI colony growth in KRAS mutant cells (Fig. 1C). Together, these findings indicate that the SUMO pathway is critical for KRAS-driven transformation growth.

To ensure the Ubc9 knockdown phenotype is on-target and to test whether the E2 ligase activity of Ubc9 is required for clonogenic growth of KRAS mutant cells, we generated HA-tagged WT and the C93A catalytically inactive mutant Ubc9 cDNAs (17) that are resistant to shUbc9#4. Western blot confirmed that the re-expression of WT Ubc9, but not the C93A mutant, was able to rescue global SUMOylation in shUbc9#4 transduced cells (Fig. S1D). Accordingly, WT Ubc9 but not mutant Ubc9 was able to rescue both AD and AI colony growth in KRAS mutant DLD-1 and HCT116 cells (Fig. 1D and Fig. S1E). Thus, the ligase activity of Ubc9 is essential for both 2D and 3D clonogenic growth of KRAS mutant cells. Because shUbc9#4 can be fully rescued and is therefore on-target, we primarily used this hairpin for the rest of our study to investigate the role of Ubc9 in KRAS mutant cells.

UBE2I/Ubc9 is an essential gene in mammals and *Ubc9*-null murine embryonic fibroblasts suffer from mitotic catastrophe (18). The SUMO pathway also plays a critical role in DNA damage response (10). We measured Ubc9 knockdown and cell cycle distribution at 2 and 5 d after shRNA transduction, and found minimal cell cycle perturbations in DLD-1 cells regardless of their KRAS status (Fig. S2A and B). Depletion of SAE1 or Ubc9 also did not trigger significant histone H2AX phosphorylation (Fig. S2C), indicating a lack of DNA damage. Thus, the difference in phenotype between Ubc9-null and Ubc9-knockdown can be attributed to the latter being a hypomorphic phenotype where residual levels of SUMOylation following Ubc9 knockdown (Figs. S1B and S2A) could still sustain cell cycle but is insufficient to support clonogenic growth.

We next tested whether Ubc9 is required for acute cell transformation by mutant KRAS. We introduced a tetracycline-inducible KRAS^{G12V} cDNA into nontransformed KRAS WT DLD-1 cells

and immortalized human mammary epithelial (HMEC-TLM) cells (Fig. S3A). In these cells, doxycycline induction of KRAS^{V12} drove AI colony growth (Fig. S3B), which was abrogated upon Ubc9 knockdown (Fig. 2A). Thus, Ubc9 is necessary for KRAS transformation. To examine whether Ubc9 is also required for transformation by other oncogenes, we first tested DLD-1 and HCT116 isogenic cell lines that are mutant or WT with respect to the *PIK3CA* oncogene (19). Because these cell lines all harbored KRAS mutations, Ubc9 depletion significantly inhibited their AD colony formation (Fig. S3C), although their *PIK3CA* status conferred no difference in their response to Ubc9 depletion. We also generated HMEC-TLM cell lines stably expressing the *EGFR*^{L858R} and *BRAF*^{V600E} oncogene (Fig. S3D). In these cells, Ubc9 depletion had a stronger inhibitory effect on the BRAF mutant colonies than the EGFR mutant colonies (Fig. S3E). To assess whether KRAS mutant CRC cells are generally more dependent on the SUMO pathway for 2D and 3D colony growth, we examined the effects of Ubc9 knockdown in a panel of CRC cell lines. As we previously reported, KRAS mutant CRC cells are dependent on KRAS for viability, whereas KRAS WT cells are not (20) (Fig. S3F). Ubc9 knockdown in CRC cells (Fig. S3G) had only modest effect on cell viability (Fig. S3H), but strongly suppressed AD colony growth of KRAS mutant cells compared with KRAS WT cells (Fig. 2B). Again, in the KRAS mutant CRC cell lines the inhibitory effect of shUbc9 is on-target and can be rescued by WT Ubc9 cDNA (Fig. S3I). Furthermore, we observed a good correlation between the cell lines' dependency on KRAS for viability and their dependency on Ubc9 for AI colony growth (Fig. 2C). All of the CRC lines, except LS123 and CaCO-2, were able to form AI colonies under 3D soft agarose conditions with reproducible efficiency. Ubc9 depletion strongly inhibited AI colony growth of KRAS mutant cells and less so in the KRAS WT cells (Fig. 2D). We again observed a correlation between the cell lines' dependency on KRAS and their dependency on Ubc9 for AI colony growth (Fig. 2E). Together, these results support the notion that KRAS mutant CRC cells are more dependent on Ubc9 for transformation growth. Of note, The KRAS WT cell lines LS411N and RKO both harbor activating *BRAF*^{V600E} mutations, yet LS411N is insensitive to Ubc9 depletion whereas RKO is sensitive in the AI colony assay. Thus, a subset of BRAF mutant cell lines might also be sensitive to Ubc9 depletion for their clonogenic growth. KRAS mutations also occur at high frequencies in lung adenocarcinomas. We tested four nonsmall CLC cell lines that are either KRAS mutant (H2030 and A549) or KRAS WT (H520 and H1838). Again we observed correlation between their sensitivities to KRAS and Ubc9 knockdown for colony growth (Fig. S3J and K).

To test whether Ubc9 is required for KRAS tumor growth in vivo, we established KRAS mutant DLD-1 cells and KRAS WT SW48 cells that stably express shUbc9#4 under the control of a tet-inducible promoter. In vitro treatment with doxycycline led to a dose-dependent knockdown of Ubc9 protein (Fig. S4A). Following injection into NOD/SCID mice and tumor establishment, we induced the Ubc9 shRNA with doxycycline-containing chow. Induction of Ubc9 shRNA, but not a control shRNA, significantly attenuated the growth of DLD-1 tumors (Fig. 3A). However, Ubc9 shRNA had little effect on the growth of SW48 tumors (Fig. 3B). This difference was not because of a lack of Ubc9 knockdown in SW48 tumors, as Western blot confirmed sustained Ubc9 knockdown in both DLD-1 and SW48 tumors (Fig. 3C and D). Immunohistochemistry revealed that Ubc9 knockdown inhibited proliferation (Ki-67 staining) and induced apoptosis (cleaved caspase-3 staining) in DLD-1 tumors but not in SW48 tumors (Fig. 3E and F and Fig. S4B). Together, these results indicate that the growth of established KRAS mutant CRC tumors is sensitive to Ubc9 depletion.

Fig. 2. Ubc9 is required for KRAS transformation. (A) Ubc9 depletion suppresses acute cellular transformation driven by KRAS^{V12}. Exogenous expression of KRAS^{V12} under a tet-inducible promoter in the otherwise non-transformed DLD-1 KRAS WT cells and HMEC-TLM cells induces AI colony growth in soft agarose. Transduction with Ubc9 shRNA strongly suppressed AI colonies ($*P < 0.05$ compared with shCtrl). (B) In a panel of colorectal cancer cell lines, KRAS mutant cells are more dependent on Ubc9 for AD colony growth than KRAS WT cells ($**P < 0.01$). (C) Correlation between dependency on KRAS for cell viability and dependency on Ubc9 for AD colony growth among CRC cell lines. Normalized cell viability following KRAS knockdown is plotted against normalized AD colony number following Ubc9 knockdown. (D) KRAS mutant CRC cells are more dependent on Ubc9 for AI colony growth ($*P < 0.05$). (E) Correlation between dependency on KRAS and dependency on Ubc9 for AI colony growth CRC cell lines. Normalized AI colony numbers following KRAS knockdown are plotted against normalized AI colony numbers following Ubc9 knockdown. Error bars represent SEM of three independent experiments.

Distinct Roles of SUMO Protein Isoforms in the Clonogenic Growth of KRAS Mutant Cells. We next sought to understand the mechanism by which KRAS mutant cells are dependent on the SUMO pathway for 2D and 3D colony growth. This dependence is not because of differential levels of Ubc9 or global SUMOylation in KRAS mutant cells as Ubc9 expression level and shRNA knockdown efficiency (Fig. S2D) and global levels of SUMO1- and SUMO2-conjugated proteins are similar between isogenic KRAS mutant and WT cells (Fig. S2A; see also Fig. S8A). Recent studies suggest that in *Drosophila* embryos the SUMO pathway is required for optimal Ras/MAPK pathway activation (21) and in mammalian cells SUMOylation of MEK inhibits ERK activation (22). Depletion of Ubc9 or SAE1 did not alter the levels of KRAS protein or phospho-ERK in KRAS mutant DLD-1 cells (Fig. S2E); thus, the SUMO pathway is likely to support the clonogenic growth of KRAS mutant cells through a MAPK-independent mechanism.

Fig. 3. Ubc9 supports KRAS mutant tumor growth in vivo. (A and B) Inducible knockdown of Ubc9 attenuated the growth of established KRAS mutant DLD-1 tumors (A) but not KRAS WT SW48 tumors (B). Cell lines expressing tet-inducible shCtrl or shUbc9#4 were injected subcutaneously into mice. Tumors were allowed to reach ~50 mm³ before the commencement of doxycycline treatment. (*P < 0.05 compared with respective –dox control. *n* = 7 for DLD-1 shUbc9 +dox group, *n* = 10 for all other groups. Tumor volume was normalized to its respective initial volume at the onset of doxycycline treatment.) (C and D) Western blot confirmation of sustained Ubc9 knockdown in tumors at the end of the experiment. Four tumors (T1–T4) from each cohort were analyzed. Numbers below Western blot indicate relative levels of Ubc9 proteins normalized to vinculin loading control. (E and F) Ubc9 knockdown decreases proliferation and increases apoptosis in DLD-1 but not SW48 tumors. Immunohistochemistry staining of Ki-67 (E) and cleaved caspase-3 (CC-3) (F) were quantitated in four representative tumors from each group (*P < 0.05 compared with respective –dox controls). Error bars represent SEM of three independent experiments.

Because Ubc9 is the sole SUMO E2 ligase that mediates the conjugation of all SUMO proteins, we tested the contribution of individual SUMO isoforms in the clonogenic assay. Human SUMO2 and SUMO3 are highly homologous and are thought to be functionally redundant, whereas SUMO1 is more divergent in sequence and is thought to have distinct functions (9). Of note, poly-SUMOylation has been attributed to SUMO2/3, whereas SUMO1 can only form mono-SUMOylation or cap SUMO2/3 chains. We thus investigated whether SUMO1 and SUMO2/3 might play functionally distinct roles in the clonogenic growth of KRAS mutant cells. We identified siRNAs against SUMO1, -2, and -3 that can effectively deplete these proteins. Because antibodies cannot distinguish SUMO2 and SUMO3 as a result of their high degree of homology, we mixed SUMO2 and SUMO3 siRNAs to codeplete both proteins (Fig. 4A). SUMO1 depletion modestly reduced the viability of KRAS mutant and WT DLD-1 cells to a similar extent (Fig. S5A), and reduced their AD colony

size to a comparable extent (Fig. 4B). In contrast, SUMO2/3 depletion had little effect on cell viability (Fig. S5A), and selectively reduced AD colony size in KRAS mutant cells (Fig. 4B). We next constructed SUMO shRNAs and assayed their effects in AI growth of KRAS mutant DLD-1 cells. Depletion of SUMO1 or SUMO2 strongly reduced AI colony numbers, and their combined depletion resulted in near-complete suppression of AI growth (Fig. 4C). Because our SUMO siRNAs and shRNAs target the 3'UTR, we carried out rescue experiments with SUMO1 and SUMO2 cDNAs (Fig. S5B and C). Interestingly, in AD colony assays SUMO1 cDNA overexpression could rescue SUMO1 siRNA but not SUMO2/3 siRNAs, whereas SUMO2 cDNA could rescue against SUMO2/3 siRNAs but not SUMO1 siRNA (Fig. 4D). Similar results were observed in AI colony assays: SUMO1 cDNA rescued SUMO1 shRNA but not SUMO2/3 shRNA combination, whereas SUMO2 cDNA fully rescued SUMO2/3 shRNAs and partially rescued SUMO1 shRNA (Fig. 4E). These

Fig. 4. Distinct roles of SUMO isoforms in the clonogenic growth of KRAS mutant cells. (A) Western blot verification of SUMO1 protein knockdown by siSUMO1#3 and SUMO2/3 protein knockdown by a mixture of siSUMO2#5 and siSUMO3#3 siRNAs. (B) AD colony assay of DLD-1 KRAS isogenic cells transfected with siSUMO1 and siSUMO2/3 mix. Aggregate colony area was quantified as these siRNAs primarily reduced colony size in the assay ($P < 0.05$). (C) AI colony assay of DLD-1 KRAS mutant cells transfected with shSUMO1, shSUMO2, and shSUMO3 either individually or in combinations ($*P < 0.05$, $**P < 0.01$ compared with shCtrl). (D) AD colony assay of DLD-1 KRAS mutant cells with SUMO1 or SUMO2 cDNA rescue following siRNA transfection ($*P < 0.05$; n.s., not significant). (E) AI colony assay of DLD-1 KRAS mutant cells with SUMO1 or SUMO2 cDNA rescue following shRNA transduction ($*P < 0.05$, $**P < 0.01$). Error bars represent SEM of three independent experiments.

results indicate that SUMO1 and SUMO2/3 play functionally distinct roles in the clonogenic growth of KRAS mutant cells, and SUMO2/3 are more critical for the clonogenic growth, but not viability, of KRAS mutant cells.

Identification of Differentially SUMOylated Proteins in KRAS Mutant

Cells. Because depletion of Ubc9 did not directly impact signaling through the Ras/MAPK pathway, we hypothesized that Ubc9 dependency could represent a form of nononcogene addiction in KRAS mutant cells where this pathway sustains the transformation growth of KRAS mutant cells through a previously unknown mechanism. To explore this possibility, we used SILAC (stable isotope labeling by amino acids in cell culture) mass-spectrometry (MS) (12) to compare global patterns of protein SUMOylation between isogenic KRAS mutant and WT cells in an unbiased fashion (Fig. S64). We focused on SUMO2-conjugated proteins because our data suggest SUMO2 is more selectively required for clonogenic growth rather than cell viability of KRAS mutant cells. To facilitate MS analysis, we established stable DLD-1 and HCT116 cell lines expressing 6xHis-tagged SUMO2 proteins to enable efficient purification of SUMOylated proteins under denaturing conditions (12). We also established cell lines expressing nonconjugatable SUMO2AA mutants as negative controls (23), and cell lines expressing a trypsin-cleavable SUMO2 Q88R mutant to facilitate SUMO site identification (24) (Fig. 5A).

Our SILAC study identified a combined total of 1022 SUMO2-conjugated proteins from all cell lines. Among these proteins, we observed enrichment for nucleic acid binding proteins and RNA

processing factors (Fig. S6B). We also identified a small number of proteins whose SUMOylation levels were either up- or down-regulated in KRAS mutant cells compared with the respective KRAS WT controls. We narrowed these down to 40 candidate “KRAS-associated SUMOylated proteins” (KASPs) that showed either consistent changes in SILAC ratios in both DLD-1 and HCT116 isogenic pairs or strong changes in SILAC ratios in one isogenic pair (Fig. 5B and Table S1). These KASP candidates also showed enrichment for nucleic acid and RNA binding proteins (Fig. S6C). We next applied ingenuity pathway analysis (IPA) to these KASPs and found that many of them can be functionally connected to the Ras hub, either directly or indirectly, through an unsupervised network analysis (Fig. 5C). Gene-set enrichment analysis of KASP candidates in 231 lung, colorectal, and pancreatic cancer cell lines from the Cancer Cell Line Encyclopedia database (25) found higher expression of KASP genes in KRAS mutant cells (Fig. S6D). In a cohort of lung cancer patients whose tumors harbored KRAS mutations (26), a higher score for KASP gene signature expression is associated with shorter patient survival (Fig. S6E). Together, these analyses suggest that KASP proteins have the potential to functionally contribute to the development of KRAS mutant tumors.

KASPs Collectively Sustain AI Growth in KRAS Mutant Cells. SUMOylation often leads to activation of target proteins, and in DNA damage repair and heat-shock response hyper-SUMOylation of substrates serves a cytoprotective role (10, 12). We reasoned that the overexpression of KASP candidates might functionally compensate

Fig. 5. Alteration in protein SUMOylation patterns in KRAS mutant cells. (A) Verification of His-tag SUMO2 affinity purification. Cell lysates from DLD-1 cells stably expressing 6×His-tagged SUMO2, SUMO2Q88R (cleavable mutant), and SUMO2AA (nonconjugatable mutant) were subject to Ni-NTA beads pull-down and eluted proteins were probed with SUMO2 antibody. (B) Differentially SUMOylated proteins in DLD-1 and HCT116 KRAS isogenic cell lines. Hits were selected from two independent SILAC experiments with isogenic DLD-1 and HCT116 cell lines. We referred to these proteins as KASPs. (C) Un-supervised molecular pathway analysis of KASPs with IPA revealed that a significant number of KASPs (blue color) could be functional connected to the Ras node.

for the reduction in their SUMOylation upon Ubc9 knockdown, and thus tested whether this could provide a partial rescue against shUbc9 in KRAS mutant cells. Of the 10 KASP candidates tested, 3 offered some degree of partial rescue in AI colony assay (Fig. 6A and Fig. S7A). They are the transcription corepressor KAP1 (27, 28), the eukaryotic translation initiation factor 3 complex subunit EIF3L (29), and the chromatin remodeling factor CHD1 (30). We confirmed that these three proteins are hyper-SUMOylated in KRAS mutant DLD-1 cells relative to KRAS WT DLD-1 cells. IP-Western blot revealed that KAP1 migrated as several higher molecular weight species, consistent with its reported poly-SUMOylation (27, 28), and the level of SUMOylated KAP1 is higher in KRAS mutant DLD-1 and HCT116 cells compared with their KRAS WT counterparts (Fig. 7A). Similarly, EIF3L and CHD1 show higher levels of SUMOylation in KRAS mutant DLD-1 cells (Fig. 6B and C).

To investigate whether the AI colony growth of KRAS mutant cells is supported by multiple KASPs, we coexpressed CHD1, EIF3L, and KAP1 in DLD-1 KRAS mutant cells in various combinations (Fig. S7B). In four KRAS mutant CRC cell lines—DLD-1, Lovo, SW1116, and SW620—the combined expression of these proteins conferred an additive rescue effects compared with individual expression, and coexpression of all three KASPs offered the best rescue (Fig. 6D). These results thus suggest that AI growth of KRAS mutant cells is likely to depend on the functions of multiple SUMOylation proteins. The lack of complete rescue by these KASPs suggests that either their overexpression was inadequate to compensate for the loss of their SUMOylation under Ubc9-depleted conditions, or additional SUMO substrates are required to fully support AI growth.

SUMOylation of KAP1 Supports AI Growth of KRAS Mutant Cells. We next examined the functional importance of KAP1 SUMOylation for sustaining AI colony growth in KRAS mutant cells because the SUMOylation sites on KAP1 have been well characterized (27, 28). KAP1 is a transcription corepressor that is recruited to its target genes by KRAB zinc finger proteins (31); it in turn recruits a transcriptional repression machinery to inhibit the expression of a large number of genes. Importantly, KAP1 is SUMOylated on key lysine residues, K554, K779, and K804, and SUMOylation enhances its repressor activity (27, 28, 32). We found basal KAP1 SUMOylation is elevated in KRAS mutant DLD-1 and HCT116 cells under normal 2D growth conditions, and KAP1 SUMOylation became further induced in KRAS mutant cells when they were placed in a low-attachment plate for 12 h to mimic the initial conditions of

AI growth (Fig. 7A). Global SUMOylation levels are unchanged in KRAS mutant cells under either attached or suspension conditions (Fig. S8A); thus, the hyper-SUMOylation of KAP1 under these conditions was not simply the result of a general up-regulation of SUMO pathway activities. It has been shown that phosphorylation of S824 on KAP1 is inhibitory for its SUMOylation (32). Accordingly, S824 phosphorylation on KAP1 is reduced in KRAS mutant cells compared with KRAS WT cells (Fig. S8B).

To test the hypothesis that KAP1 SUMOylation supports the clonogenic growth of KRAS mutant cells, we generated several FLAG-tagged KAP1 mutants, including a KAP1 3KR mutant, with the three major SUMOylation sites, K554, K779, and K804, mutated to arginine, a SUMO1-fused KAP1 (S-WT) that mimics activated KAP1 and promotes its poly-SUMOylation (32), and a SUMO1-fused 3KR (S-3KR) mutant that mimics mono-SUMOylated KAP1. Functionally, both WT and S-WT KAP1 could undergo poly-SUMOylation, whereas neither the 3KR nor the S-3KR mutants were poly-SUMOylated, although the 3KR mutant can still undergo mono-SUMOylation (Fig. S8C). We generated stable KRAS mutant and WT DLD-1 cells that expressed each of these constructs to similar levels (Fig. S8D) and tested whether each mutant could functionally rescue Ubc9 knockdown in AD and AI colony assays. In AD colony assay with KRAS mutant DLD-1 cells, only WT and S-WT KAP1, but not the 3KR and S-3KR mutants, provided partial rescue (Fig. 7B). This finding indicates that only poly-SUMOylated KAP1 can functionally contribute to the clonogenic growth of KRAS mutant cells. Similarly, WT and S-WT KAP1, but not the 3KR mutant, also provided partial rescue against Ubc9 knockdown in the AI colony assay (Fig. 7C).

We next tested whether KAP1 is required for the clonogenic growth of KRAS mutant cells. We identified several KAP1 shRNAs that effectively depleted KAP1 protein. KAP1 knockdown by two independent shRNAs that effectively depleted KAP1 protein (shKAP1#5 and shKAP1#7) (Fig. S8E) did not significantly impair the viability of isogenic DLD-1 and HCT116 cells under normal 2D growth conditions (Fig. S8F). However, KAP1 knockdown selectively reduced the size of AD colonies (Fig. 7D and Fig. S8G) and the number of AI colonies in KRAS mutant DLD-1 and HCT116 cells (Fig. 7E). Because the hairpin shKAP1#7 targets the 3'UTR, we attempted rescue experiment with various KAP1 cDNAs (Fig. S8H). Consistent with our conclusion that poly-SUMOylated KAP1 is required for clonogenic growth, only the WT and S-WT KAP1, but not the 3KR mu-

Fig. 6. KASP candidates KAP1, EIF3L, and CHD1 support AI growth of KRAS mutant cells. (A) Identification of KASP cDNAs whose stable overexpression can partially rescue AI colonies in KRAS mutant DLD-1 cells upon Ubc9 knockdown. Colony numbers were normalized to control shRNA (shCtrl) transduced cells (* $P < 0.05$ compared with shUbc9#4 alone). (B) EIF3L SUMOylation is elevated in KRAS mutant DLD-1 cells. DLD-1 mutant and WT cells stably expressing HA-tagged EIF3L were subject HA antibody IP and probed with SUMO2 antibody. (C) CHD1 SUMOylation is increased in KRAS mutant DLD-1 cells. DLD-1 mutant and WT cells stably expressing HA-tagged CHD1 and His-tagged SUMO2 were subject Ni-NTA beads pull-down and SUMO-conjugated CHD1 was probed using HA antibody. The relative levels of HA-CHD1 in the SUMO pull-down were quantified below the Western blot (n.d., not detectable). (D) Rescue of AI colony growth in 4 KRAS mutant CRC cell lines following Ubc9 knockdown by CHD1, EIF3L, and KAP1 cDNAs. Combined expression of these proteins provides additive rescue effect. All AI colony numbers were normalized against shCtrl (* $P < 0.05$ and ** $P < 0.01$ compared with shUbc9#4 alone).

tant, were able to functionally rescue colony growth against shKAP1#7 in KRAS mutant DLD-1 cells (Fig. 7F). Taken together, these results support the notion that KAP1 poly-SUMOylation functionally contributes to AD and AI colony growth in KRAS mutant cells.

Discussion

The prevalence of KRAS mutations in cancer and the lack of effective therapy make it a high priority to discover new approaches to target these cancers. Difficulties in druging KRAS itself and the limited efficacy of inhibitors targeting Ras effector kinases have motivated us to identify alternative targets using synthetic lethal approaches. Through this endeavor, we discovered the SUMO pathway—and its E2 ligase Ubc9 in particular—to play a critical role of in KRAS-driven transformation. Our finding thus suggests Ubc9 could be a useful target in KRAS mutant cancer.

Although the ubiquitin pathway has been extensively studied in cancer, the role of the SUMO pathway in cancer is much less well understood. In this study we show that depletion of the SUMO E2 ligase Ubc9, the E1 ligase subunit SAE1, and SUMO2/3 all selectively impaired AD and AI clonogenic growth of KRAS mutant CRC cells. We further demonstrate that KRAS mutation is associated with elevated SUMOylation of a subset of cellular proteins, including KAP1, CHD1, and EIF3L, and these proteins collectively supported the AI growth of KRAS mutant cells. Based on these observations, we propose a model where KRAS-driven transformation requires the engagement of the SUMO pathway to maintain proliferation under the otherwise restrictive condition of anoikis. KRAS and Ubc9 do so, in part, by up-regulating the SUMOylation of a subset of proteins we collectively termed KASPs (Fig. 7G). Because SUMOylation of multiple KASPs are altered by the KRAS oncogene, it is likely

that they collaboratively contribute to the transformation growth of KRAS mutant cells. This finding is consistent with the observation that combined overexpression of KASPs afforded better rescue of AI growth against Ubc9 depletion than each protein individually. In addition to mutant KRAS, we observed that the clonogenic growth of two BRAF mutant cell lines (RKO and HMEC-TLM-BRAF^{V600E}) are also sensitive to Ubc9 depletion. Thus, the SUMO pathway is likely to be critical for transformation driven by oncogenic activation of the Ras/Raf pathway.

The molecular mechanism by which the SUMO pathway supports AI growth requires further elucidation. Global SUMOylation levels between KRAS mutant and WT DLD-1 cells are comparable under attached conditions; and global SUMOylation in KRAS mutant DLD-1 cells do not further increase when cells were placed under suspension conditions. Instead, we found that a small number of cellular proteins, including KAP1, CHD1, and EIF3L, are hyper-SUMOylated in KRAS mutant cells, and in the case of KAP1, its SUMOylation is further elevated in KRAS mutant cells under AI conditions. This finding is analogous to the DNA damage response where a subset of DNA repair proteins became activated by SUMOylation (10). Furthermore, like the DNA damage response where the coordinated SUMOylation of multiple proteins are necessary to execute repair, our data indicate that the coordinated SUMOylation of multiple KASPs are necessary to support AI growth. Interestingly, we found that the 2D proliferation of KRAS mutant cells is less sensitive to SUMO2/3 depletion, whereas their clonogenic growth is highly dependent on SUMO2/3. This finding is consistent with the observation that poly-SUMOylation of KAP1 (which is mediated through SUMO2/3), but not mono-SUMOylation of KAP1, is required for its role in AI growth. Because Ubc9 is the sole E2 ligase, it is likely that specific SUMO E3s and SENPs are

Fig. 7. Poly-SUMOylated KAP supports clonogenic growth of KRAS mutant cells. (A) KAP1 SUMOylation is elevated in KRAS mutant cells and becomes further increased upon loss of cell attachment. Cell lysates from DLD-1 and HCT116 KRAS mutant and WT cells under normal attached conditions (att) or plated in suspension condition (susp) for 12 h were subject to KAP1 IP followed by SUMO2 Western blot. (B) Rescue of AD colony in DLD-1 KRAS mutant cells following Ubc9 knockdown by various KAP1 cDNAs: 3KR, KAP1 with K554/K779/K804 mutated to R; S-WT, KAP1 with SUMO1 fused to the N terminus; S-3KR, KAP1-3KR mutant with SUMO1 fused to the N terminus ($P < 0.05$). (C) Rescue of AI colony in DLD-1 KRAS mutant cells following Ubc9 knockdown by various KAP1 mutants ($P < 0.05$). (D) KAP1 knockdown impairs AD colony growth in KRAS mutant DLD-1 and HCT116 cells. Aggregate colony area was quantified as KAP1 shRNAs primarily reduced colony size in this assay ($P < 0.05$). (E) KAP1 knockdown reduced AI colony number in KRAS mutant DLD-1 and HCT116 cells ($P < 0.05$). (F) WT and S-WT KAP1, but not 3KR or S-3KR mutant KAP1, rescued AD colony growth in DLD-1 mutant cells against shKAP1#7. (G) A proposed model for the role of Ubc9 and KASPs in supporting the transformation growth of KRAS mutant cells. Error bars represent SEM of three independent experiments.

selectively engaged in KRAS mutant cells to target KASPs, and further studies are necessary to delineate the molecular mechanism by which the selective hyper-SUMOylation of KAP1, CHD1, and ELF3L occurs in KRAS mutant cells. One potential mechanism is that the Ras/Raf/MAPK kinase pathway might selectively phosphorylate and regulate one or more SUMO E3 ligases that engage these KASPs. Of note, KAP1 possesses E3 activity for auto-SUMOylation (27); thus, its E3 activity might be stimulated both by mutant KRAS and by loss of attachment. Given the enrichment of DNA/RNA binding proteins among KASPs, we speculate that the SUMO pathway might coordinate a transcriptional program that is necessary for KRAS mutant cell proliferation and survival under AI conditions.

Ubc9 is an essential protein because homozygous deletion of the mouse *Ube2i* gene is lethal (33) and deletion of Ubc9 in mouse embryonic fibroblasts leads to mitotic defects (18). Our Ubc9 knockdown experiments, on the other hand, revealed a hypomorphic phenotype that is distinct from the null phenotype.

We found that an ~80% depletion of Ubc9 or SAE1, and a significant reduction in global SUMOylation, is tolerated in both KRAS mutant and WT cells. This finding suggests that under normal growth conditions, the SUMO pathway is not fully engaged and a small amount of Ubc9 activity is sufficient to fulfill its role in the cell cycle. On the other hand, a reduction in Ubc9 activity is insufficient to maintain the clonogenic proliferation of KRAS mutant cells. Clonogenic growth, particularly under AI conditions, is inhibited in normal cells as they undergo anoikis upon loss of attachment (34). The *KRAS* oncogene can override this tumor-suppressor mechanism. Cellular adaptation underlying transformation is not fully understood, but it likely involves sustained mitogenic signaling and suppression of growth inhibitory and apoptotic signals. Our data indicate that KRAS transformation is dependent on the SUMO pathway, and this could reflect a form of nononcogene addiction in these cells (8).

Both Ubc9 and the E1 ligase SAE1/UBA2 are druggable enzymes. Our cDNA rescue experiments demonstrate that the

catalytic activity of Ubc9 is critical for KRAS transformation, thus suggesting small molecule inhibitors against Ubc9 might be effective at attenuating the growth of KRAS mutant tumors. Of note, it has been reported that Ubc9 can be targeted by small molecule inhibitors (35). Although inhibition of Ubc9 is likely to perturb the function of a large number of cellular processes, a therapeutic window could exist for those cancer cells exhibiting nononcogene addiction to this pathway. The anticancer effects of inhibitors against the proteasome and the mammalian target of rapamycin pathway demonstrate that essential proteins that regulate broad cellular functions can be successfully exploited for cancer therapy. Analogous to this finding, SUMO ligase inhibitors could prove useful as cancer drug targets.

Materials and Methods

Cell Culture. The *KRAS* and *PIK3CA* isogenic DLD-1 and HCT116 cell lines were a gift from Bert Vogelstein, The Johns Hopkins University Medical School, Baltimore (36) and their use for *KRAS* synthetic lethal analysis has been described before (7). Briefly, the parental DLD-1 and HCT116 cells harbor heterozygous *KRAS* mutations (referred to as *KRAS* mutant DLD-1 and HCT116 cells). Knocking-out the mutant *KRAS* allele by gene-targeting generated the *KRAS* WT DLD-1 and HCT116 cells, respectively (4, 36). The *PIK3CA* isogenic DLD-1 and HCT116 cell lines were generated in a similar fashion (19). Because the parental DLD-1 and HCT116 cell lines harbor *KRAS* mutations, all of the *PIK3CA* isogenic cell lines also retained their mutant *KRAS* allele. The colorectal cancer cell lines SW48, SW620, SW403, SW1116, Lovo, LS123, LS411N, RKO, and CaCo-2 were cultured in McCoy's 5A media supplied with 10% (vol/vol) FBS and antibiotics. The human mammary epithelial cell line HMEC-TLM was described previously (37) and was cultured in MEGM media (Lonza). The non-small CLC cell lines H2030, A549, H520, and H1838 were cultured in RPMI-1640 media supplied with 10% (vol/vol) FBS and antibiotics. For proliferation assays, cells were seeded in 96-well plates on day 0, either transfected with siRNA using RNAiMAX (Invitrogen) or transduced with retroviral shRNA on day 1, and cell viability was measured with CellTiterGlo (Promega) on day 6. For adherent colony assays, 1 d after shRNA transduction, cells were seeded in six-well plates at 200 cells per well and colony numbers were determined with Coomassie blue staining 8–10 d later. For anchorage-independent colony assays, 500–2,000 cells were seeded in each well of six-well plates in media containing 0.35% low-melting agarose and colonies were visualized with crystal violet staining on day 20. For tetracycline-inducible expression of shRNA or cDNA, cells were transduced with virus and selected with puromycin (1 μ g/mL) for 3 d before doxycycline induction. For proliferation or colony assays, the dox⁺ cells were cultured in the presence of doxycycline at indicated concentrations.

Molecular Biology, Plasmids, and Reagents. Sequence information for shRNAs, siRNAs and primers are in Tables S2 and S3. A control shRNA (shCtrl) targeting firefly luciferase was used as negative control. The shRNAs were expressed using the retroviral MSCV-PM vector (38). cDNAs for Ubc9, SUMO1, and SUMO2 were PCR-cloned with Gateway vectors (39). Ubc9 cDNA was expressed in retroviral MSCV-puro vectors. Ubc9 WT and C93A cDNAs resistant to Ubc9 shRNA#7 were generated by QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) with primers 5'-cctattaggaatacAgaGTtGtGaaCgaacaaatattcc-3' and 5'-ctctcgaggacagtGcCctgtccatcttagag-3', respectively. SUMO2 Q88R cDNA was generated based on previous reports (24). All KASPs cDNAs except KAP1 were cloned into pHAGE vector with Gateway system. WT and mutant KAP1 cDNAs were gifts from D.K.A. and were expressed using pHAGE vectors. Tetracycline-inducible *KRAS* cDNA, and shUbc9#4 and shCtrl were generated based on pINDUCER10 vectors (40). N-HA tagged mutant EGFP^{L858R} and BRAF^{V600E} cDNAs were expressed using the MSCV vector (Clontech). Antibodies were from the following sources: Ubc9, SAE1, and SUMO2/3 (Abcam); SUMO1 (GeneTex); *KRAS* (Sigma Aldrich); phospho-ERK and phospho-AKT (Cell Signaling Technologies); and KAP1 and phospho-KAP1 (Bethyl Laboratories).

For immunoprecipitation, 1 $\times$ 10⁷ DLD-1 *KRAS* isogenic cells were plated in regular (adherent) or low attachment (suspension) cell culture plates for 12 h, collected, and lysed with Nonidet P-40 lysis buffer (50 mM Tris at pH 7.4, 150 mM NaCl, 1% Nonidet P-40, protease inhibitor mixture, and 10 mM *N*-ethylmaleimide which serves to block SUMO peptidases). Lysates were incubated with 1- μ g antibody for 1 h and then 30- μ L protein A/G agarose beads (Pierce) overnight. Beads were rinsed with lysis buffer three times and eluted with SDS/PAGE sample buffer.

For reverse-transcription quantitative PCR (RT-qPCR) analysis, RNAs were extracted with RNeasy mini kit (Qiagen) and reverse-transcribed to cDNA with

a high-capacity cDNA reverse-transcription kit (Applied Biosystems). Real-time PCR was performed with Sybr green mix and indicated primers on the ABI 7900 HT real-time thermal cycler. mRNA levels of each gene were normalized to that of GAPDH.

Purification of SUMOylation Proteins and SILAC MS. For profiling SUMOylation changes in *KRAS* isogenic DLD-1 and HCT116 cells, cells were stably transduced with pHAGE-6xHis-SUMO2 vector. *KRAS* mutant cells were cultured in isotope-labeling DMEM media (with ¹³C6 L-Lysine and ¹³C6, ¹⁵N4 L-Arginine, from Pierce) (heavy media) for 10 passages, and *KRAS* WT cells in normal DMEM media (light media). Cell lysates from heavy- and light-media-cultured cells were mixed at a ratio of 1:1 and SUMOylated proteins were purified with nickel-agarose beads (Sigma Aldrich) according to a previous report (41). Changes of SUMOylation on substrates were measured with a ratio of light/heavy peptides by MS. For SILAC MS, peptide fractions were loaded onto a Zorbax C18 trap column (Agilent) to desalt the peptide mixture using an on-line Eksigent nano-LC ultra HPLC system. The peptides were then separated on a 10-cm Picofrat Biobasic C18 analytical column (New Objective). Peptides were eluted over a 45-min linear gradient of 5–35% (vol/vol) acetonitrile/water containing 0.1% formic acid at a flow rate of 250 nL/min, ionized by electrospray ionization in positive mode, and analyzed on a LTQ Orbitrap Velos mass spectrometer (Thermo Electron). All LC-MS analyses were carried out in a "data-dependent" manner in which the top six most-intense precursor ions from the MS1 precursor scan (*m/z* 300–2,000) were subjected to collision-induced dissociation. Precursor ions were measured in the orbitrap at a resolution of 60,000 (*m/z* 400) and all fragment ions were measured in the ion trap. LC-MS/MS data were searched using the Sequest algorithm within Proteome Discoverer 1.2 (Thermo Electron). All data were searched against the Swissprot protein database (440,803 entries) for peptide and protein identifications. Trypsin was specified as the digestion enzyme, allowing for up to two missed cleavage sites. Carbamidomethylation was set as a static modification and Oxidation, SILAC ¹³C(6) ¹⁵N(4) (R), and SILAC ¹³C(6) were selected as variable modifications. Two additional variable modifications of +471.2077 Da and +477.2279 Da were also included as variable modifications to account for the addition of a GGTQQ SUMOylation tag on heavy or light lysine residues. Precursor and fragment ion mass tolerances were set to 25 ppm and $\pm$ 0.8 Da, respectively. SILAC quantitation was performed using an in-house software, QUIL (42). For each cell line, two samples were collected for SUMOylation profiling. The proteins with ratio <0.75 or >1.5 were considered as targets with up-regulated or down-regulated SUMOylation in *KRAS* mutant cells.

Xenograft Experiments. DLD-1 and SW48 cells were transduced with pINDUCER10-shCtrl or shUbc9#4 and selected with puromycin (10 μ g/mL) for stable cells. Next, 5 $\times$ 10⁶ cells were suspended in 50 μ L PBS and 50 μ L Matrigel (from BD) and injected into SCID/NOD mice in the rear flank, subcutaneously. When tumors reached $\sim$ 50 mm³ in volume, doxycycline was delivered through Teklad doxycycline diets (Harlan) and tumor volumes were measure twice a week. Mice were killed before tumors reached 22 mm in length in the longest dimension. Tumor samples were collected and stored in liquid nitrogen and DNA, RNA, and protein were extracted with an all-prep kit (Qiagen). For immunohistochemistry, formalin-fixed tumor slides were deparaffined and treated for antigen retrieval for 15 min. Immunohistochemical staining for K₆₇, cleaved-caspase 3, and phospho-histone 3 serine 10 was carried out using a proliferation/apoptosis kit (Cell Signaling Technology). Animal studies were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide.

Bioinformatics Analysis of SUMO Substrates and Statistics. Molecular functions of all SUMO substrates detected in SILAC MS were classified using the Panther software tool in the DAVID functional gene annotation tool (43). For functional analysis of KASPs, the list of 40 KASP genes were imported into the IPA tool and the default analysis was used to seek the best fit network in an unsupervised manner that would include the most KASP proteins; the result was a network shown in Fig. 5C. Expression of the KASP signature in *KRAS* mutant or WT cancer cell lines was analyzed using the Gene Set Enrichment Analysis tool (Broad Institute) and gene-expression data for 86 *Ras* mutant and 145 *Ras* WT lung, colorectal, and pancreatic cell lines were from the Cancer Cell Line Encyclopedia database (25). Survival analysis of lung cancer patients was carried out as previously described (7). For shRNA and cDNA cell viability and colony assays, results were assessed by one-sided *t* test. A *P* < 0.05 was considered statistically significant.

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