Novel AKT1-GLI3-VMP1 Pathway Mediates KRAS Oncogene-induced Autophagy in Cancer Cells

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Background: Autophagy plays a role in cancer development.

Results: Oncogenic KRAS induces Vacuole Membrane Protein 1 (VMP1) through a novel AKT1-GLI3-p300 pathway and requires VMP1 to regulate autophagy in cancer cells.

Conclusion: Define a novel pathway initiated by the oncogene KRAS regulating autophagy.

Significance: These findings contribute to the understanding of the mechanism underlying oncogene-induced autophagy.

Autophagy is an evolutionarily conserved degradation process of cytoplasmic cellular constituents. It has been suggested that autophagy plays a role in tumor promotion and progression downstream oncogenic pathways; however, the molecular mechanisms underlying this phenomenon have not been elucidated. Here, we provide both in vitro and in vivo evidence of a novel signaling pathway whereby the oncogene KRAS induces the expression of VMP1, a molecule needed for the formation of the autophagosome and capable of inducing autophagy, even under nutrient-replete conditions. RNAi experiments demonstrated that KRAS requires VMP1 to induce autophagy. Analysis of the mechanisms identified GLI3, a transcription factor regulated by the Hedgehog pathway, as an effector of KRAS signaling. GLI3 regulates autophagy as well as the expression and promoter activity of VMP1 in a Hedgehog-independent manner. Chromatin immunoprecipitation assays demonstrated that GLI3 binds to the VMP1 promoter and complexes with the histone acetyltransferase p300 to regulate promoter activity. Knockdown of p300 impaired KRAS- and GLI3-induced activation of this promoter. Finally, we identified the PI3K- AKT1 pathway as the signaling pathway mediating the expression and promoter activity of VMP1 upstream of the GLI3-p300 complex. Together, these data provide evidence of a new regulatory mechanism involved in autophagy that integrates this cellular process into the molecular network of events regulating oncogene-induced autophagy.

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The abbreviations used are: VMP1, vacuole membrane protein 1; NT, nontargeting control; ca, constitutively active; EGF, enhanced GFP; LC3, light chain 3.
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EXPERIMENTAL PROCEDURES

Cell Culture Conditions and Plasmids—PANC1, CHO, 293T, MCF10A, and HeLa cell lines were obtained from ATCC and were cultured as reported previously (18, 19). The L3.6 cells were kindly provided by Dr. Bidder (MD Anderson Cancer Center, Houston, TX) and HPDE6 cells by Dr. Tsao (University of Toronto, Toronto, Ontario, Canada). Experiments were performed using a luciferase reporter for the VMP1 promoter containing 3005 bp upstream of the transcriptional starting site. For the cloning of this regulatory sequence into the pGL3 vector (Promega, Madison, WI), we used the following primers: CGGGTGACGCTGGCCGATCCTACGG and GGGAAAGCT-TCCCGAGATCCTGGAATC. The expression vector FLAG-p300 was kindly provided by Dr. Donald Tindall (Mayo Clinic, Rochester, MN). The KRASG12D mutant was a gift from Dr. Brian Billadeau (Mayo Clinic, Rochester, MN). The EGFP-LC3 and TK-pRL Renilla vector were provided generously by Dr. Scott Kaufmann (Mayo Clinic, Rochester, MN). The shRNA targeting KRAS, AKT1, AKT2, AKT3, GLI3, VMP1, and p300 and the non-targeting control (NT) were obtained from Sigma (St. Louis, MO). The pool of shRNA targeting p300 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The GLI-luciferase reporter was provided kindly by Dr. Chi-chung Hui (University of Toronto, Toronto, Ontario, Canada). The constitutively active (ca) caPI3K, caAKT1 and dominant-negative AKT1 were kindly provided by Dr. Fidler (MD Anderson Cancer Center, Houston, TX) and Dr. Fergus Couch (Mayo Clinic, Rochester, MN), as described previously (21, 27). Briefly, DNA was harvested from mouse tails using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) and genotyped by PCR. Reaction conditions for Cre were 36 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. Conditions for kras mutant were 40 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 1 min. Mice were housed under pathogen-free conditions in the Mayo Clinic facilities. All studies were conducted in compliance with the Mayo Clinic Institutional Animal Care and Use Committee guidelines.

Transfection—PANC1, HeLa, 293T, HPDE6, and CHO cells were transfected with Lipofectamine (Invitrogen) as described previously (18). 1.6 × 10^5 cells were plated into six-well plates with standard and transfected 24 h later. For each condition, 0.6 μg of VMP1 promoter reporter or 0.4 μg of GLI-luciferase reporter was used. In overexpression assays, 1.4 μg of KRASG12D, GLI3, caAKT1, caPI3K, or p300 vectors were used. For transfection of multiple constructs, 3 μg of DNA were used, consisting of 0.6 μg of VMP1 promoter reporter and equal amounts of the other vectors. In knockdown experiments, 0.6 μg of VMP1 promoter reporter was cotransfected with 2.4 μg of shRNA. In rescue experiments, 0.4 μg of VMP1 promoter reporter was used together with 1.2 μg of expression vector and 2.4 μg of shRNA. In each set of experiments, equal amounts of plasmid DNA were used by adding empty vectors to appropriate controls. L3.6 cells were transfected by electroporation at 320 V for 10 ms (BTX, Harvard Apparatus, Holliston, MA), and MCF10A cells were transfected at 375 V for 10 ms.

 Luciferase Reporter Assay—Cells were grown and transfected as indicated above. For luciferase reporter assays, cells were plated in triplicate into six-well plates in medium containing 10% FBS. Samples were harvested and prepared for luciferase assays in accordance with the manufacturer’s protocol (Promega, Madison, WI): cells were harvested 36–48 h after transfection for overexpression studies and 72 h after transfection when performing shRNA knockdown assays. To control for intersample variations in transfection efficiency, the total protein of samples in each well was quantitated using the Bio-Rad protein assay (Bio-Rad), and luciferase readouts were normalized to protein content (Figs. 3–8) or Renilla readings (Promega) (supplemental Figs. 2–4). Relative luciferase activity represents luciferase readouts/protein concentrations or luciferase/Renilla readouts normalized to control cells within each experiment. Similar results were obtained using either normalization protocol.

Reverse Transcriptase PCR (RT-PCR)—Total RNA was extracted from cultured cells or pancreatic tissue using TRIzol reagent (Invitrogen). Moloney murine leukemia virus reverse transcriptase was used to reverse transcribe 5 μg of RNA (Invitrogen). A portion of the total cDNA was amplified by PCR using 94 °C denaturation, 58 °C annealing, and 72 °C extension temperatures for 40 cycles with Ex Taq™ DNA polymerase (Takara Bio, Inc., Otsu, Shiga, Japan). For AKT2 amplification, an annealing temperature of 60 °C was used. Sense and antisense primers used for amplification of each mRNA species were as follows: human GLI3, CCAACGGAAATCAATAGG-GTGGAAA and GAGGGTGGTGTGTTAGTAAACATTAC; mouse gl3, AACCTCACTCTGCAACAGGACAG and GTGTTTGTGGTCTCTTGTGCTAC; KRAS, GAACCCAGCAC-GTTACCTCAAAGCAG and ACACCTGAACCCAGTTAGCTCCTGTTG; p300, TGCCAGTCTACAGGCTCTACGACA and AGTCTGAGTTATCGGTGCTGAGTC; AKT1, GGCA-CAGATGCAACCTCACTATG and ACACCGGAAAGGT-TAAGGCGTCAA; AKT2, TTGGACAGATGACTGCGCACAGT and AGACACAGTCTATTGTCAC; AKT3, CAAAT; mouse GAPDH, GACCTGACCT-GCGCTTAGAAAA and ACCACCTGTGTGCTGAGC-CAAAT; mouse 18 S, AACCAGTGAACCCCATCCTGAT and CAGGTTACCTACGGAACACTTGTG. Amplified products were visualized under UV illumination after electrophoresis on ethidium bromide-stained 2% agarose gels. Amplification of the appropriate gene fragments was confirmed by comparison with molecular weight markers run on the same gel.

Real-time expression analysis of VMP1 and 18S transcript was performed using TaqMan® fluorescence methodology and ABI 7900 (Applied Biosystems, Foster City, CA). A predesigned primer/probe set for mouse and human VMP1 and 18S expression was purchased from Applied Biosystems. RNA (5 μg) was extracted from cultured cells or pancreatic tissue and was reverse-transcribed using a high capacity cDNA synthesis kit (Applied Biosystems). From each sample, 2 μl of the cDNA synthesis reaction were used for quantitative PCR analysis. The
amount of VMP1 transcript was calculated and expressed as the difference relative to the control gene 18 S (2\(^{-\Delta Ct}\), where \(\Delta Ct\) represents the difference in threshold cycles between the target and control gene).

**Chromatin Immunoprecipitation (ChIP) Assay**—Chromatin immunoprecipitation was conducted following the Magna ChIP kit protocol (EMD Millipore, Bedford, MA). Briefly, PANC1 cells (7 × 10\(^6\)) were cross-linked with 1% formaldehyde directly into the media for 10 min at room temperature. The cells were then washed and scraped with phosphate-buffered saline and collected by centrifugation at 800 g for 10 min. The pellet was then resuspended in nuclear lysis buffer and sheared to fragment DNA to ~700 bp. Samples were then immunoprecipitated using a GLI3 antibody (Santa Cruz Biotechnology), p300 antibody (Santa Cruz Biotechnology), normal goat IgG (Santa Cruz Biotechnology), or normal rabbit IgG (Santa Cruz Biotechnology) overnight at 4 °C on a rotating wheel. Following immunoprecipitation, samples were washed and eluted using the chromatin immunoprecipitation kit in accordance with the manufacturer’s instructions. Cross-links were removed at 62 °C for 2 h, followed by 10 min at 95 °C, and immunoprecipitated DNA was purified (EMD Millipore, Bedford, MA) and subsequently amplified by PCR. PCR was performed using seven primer sets for the seven areas containing potential GLI binding sites in the VMP1 promoter sequence (Fig. 5E): 1) CACTGATGGTATCCAGGGCC (sense) and CGTTCCTTCTCCAGATGTTACCG (antisense); 2) CCGTATCTATCTGGAGAGAGG (sense) and GGCAACATAGCACAGGACCAA (antisense) (antisense); 3) GCCTATGGTCTTAACTAAGGGA (sense) and AGTGGCTTAAGGTTCAGAGAGA (antisense); 4) GTCTCAATTCTCTGTCCTCAGGA (sense) and GTATCGAATGCTCTCTCCCGAAATCAG (antisense); 5) CTGACACAGAGGCTTCGACCTGTG (sense) and CATAACCTCAGCGCCATCATATG (antisense); 6) CATGATTGGCCATGCTGGGCTTATAG (sense) CCGTTCCTTCTCCAGATGTTACCG (antisense); 7) AGGCAACAGTTTCTATCGCTGGA (sense) and CTTAGTGGTTTACCTTCCCGGCT (antisense). PCR products were visualized by 2% agarose gel.

**Immunoblotting**—Western blot was performed as described previously (14, 18). 5 × 10\(^5\) PANC1, MCF10A, 293T, and HeLa cells were plated in standard medium with 10% FBS and used for the RNAi studies. LC3-II, AKT, and phospho-AKT antibodies were obtained from Cell Signaling (Danvers, MA). Anti-tubulin and β-actin antibody was obtained from Sigma-Aldrich, and anti-VMP1 was purchased from Abcam (Cambridge, MA). The p62 antibody was purchased from Santa Cruz Biotechnology. Peroxidase-conjugated secondary antibodies were used, and immunoreactive proteins were detected by chemiluminescence (GE Healthcare).

**Fluorescence Microscopy**—MCF10A, PANC1, and HeLa cells were transfected with EGFP-LC3 to identify the autophagosome. Punctate fluorescence was imaged by fluorescence microscopy using an LSM510 microscope (Zeiss, Heidelberg, Germany).

**Immunoprecipitation**—PANC1 cells were washed twice with cold phosphate-buffered saline and lysed in lysis buffer (150 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 20 mM MgCl\(_2\)) supplemented with Complete protease inhibitor tablets (Roche Applied Science, Penzberg, Germany) for 1 h on ice. After the lysates were cleared at 15,000 × g for 20 min, supernatants were collected and subjected to immunoprecipitation following the Dynabeads Protein G immunoprecipitation kit protocol (Invitrogen). The following antibodies were cross-linked to Dynabeads Protein G for 1 h at room temperature: anti-p300, anti-GLI3, and normal rabbit IgG from Santa Cruz Biotechnology. Then, Dynabeads-antibody complexes and lysates were incubated overnight at 4 °C with rotation. Proteins were eluted by addition of SDS sample buffer and incubation at 95 °C for 5 min. These eluates were subjected to Western blot analysis as described above, using anti-p300 and anti-GLI3 antibodies (Santa Cruz Biotechnology).

**RESULTS**

**KRAS Requires VMP1 to Induce Autophagy**—The GTPase KRAS is a central transducer of multiple signaling pathways involved in cell growth and survival. Mutations such as the G→A transition in codon 12 are highly prevalent in human tumors. This mutation results in a glycin to aspartic acid substitution (G12D) in the expressed protein, compromising its GTPase activity and resulting in constitutive downstream signaling and subsequent cellular transformation (19–21). Recent reports have shown that oncogenes, including KRAS, can promote autophagy in cancer cells (20–24). Using three well-established markers of this cellular process, we demonstrated that KRASG12D increases autophagy. This was shown by the increase in the punctate distribution of microtubule-associated protein light chain 3 tagged with EGFP (EGFP-LC3) in MCF10A cells transfected with oncogenic KRAS mutant (Fig. 1A). Similarly, overexpression of KRASG12D led to an increase in LC3-II, the processed form of LC3, in MCF10A, 293T, and HeLa cells (Fig. 1B). To complement these results, we determined the expression of the polyubiquitin-binding protein p62/SQSTM1, a molecule that is degraded during the progression of autophagy. As demonstrated by supplemental Fig. 1A, KRASG12D induces the degradation of p62 in HeLa cells. Conversely, knockdown of KRAS in cultured pancreatic cancer cells (PANC1) carrying the G12D oncogenic mutant of this GTPase led to a decrease in processing of LC3 (Fig. 1C) and EGFP-LC3 punctae (Fig. 1D). Expression of KRAS by RT-PCR was used as a control for the efficiency of shRNA knockdown (Fig. 1C, lower panel). In addition, we determined KRAS signaling activity by studying AKT activation using antibodies against the phosphorylated form of AKT (in cells overexpressing a mutant KRASG12D (supplemental Fig. 1B) or transfected with shRNA targeting KRAS (supplemental Fig. 1C). As expected, KRASG12D overexpression increased the phosphorylation of AKT, and the shRNA targeting this GTPase diminished the phosphorylation of this kinase. Together, these findings establish a role for KRAS in the induction and maintenance of autophagy in cancer cells. Expression studies of different components of the machinery essential for the formation of the autophagosome demon-
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FIGURE 1. A, MCF10A cells transfected with EGFP-LC3 and either KRASG12D mutant or parental control vector were analyzed by fluorescence microscopy to determine the distribution of punctate EGFP-LC3 fluorescence. The right panel shows the quantification of EGFP-LC3 positive over the total number of cells between the experimental groups. B, the indicated cells were transfected with control vector or KRASG12D. At 48 h post-transfection, LC3 and tubulin were determined by Western blot. C, PANC1 cells carrying an oncogenic mutant KRASG12D were transfected with two independent KRAS shRNA (shKRAS (1) and shKRAS (2)) constructs and NT control. The levels of LC3 lipiddation and TUBULIN were determined by Western blot. KRAS and GAPDH mRNA expression levels by RT-PCR were included as a control for the shRNA knockdown. D, punctate EGFP-LC3 distribution of PANC1 cells transfected with KRAS shRNA constructs and NT control.

Indeed, overexpression of caAKT1 was able to rescue the effect of shRNA targeting the oncogenic KRASG12D in PANC1 cells (Fig. 4D). Indeed, overexpression of caAKT1 restores the activity of the VMP1 promoter in cells transfected with shRNA targeting KRAS. Taken together, these findings suggest the PI3K-AKT1 axis as a mediator of KRAS-induced VMP1 expression.

KRAS-GLI3 Pathway Couples Membrane Signals to Transcriptional Activation of VMP1—Initial bioinformatics analysis and promoter deletion studies have identified GLI proteins (GLI1, GLI2, and GLI3) as candidate mediators of KRAS-PI3K-AKT1 activation of the VMP1 promoter in cancer cells (data not shown). These transcription factors are known effectors of the Hedgehog pathway and essential for regulation of the cellular functions mediated by this signaling cascade (28, 29). GLI1 and GLI2 function predominantly as activators, whereas GLI3 is thought to act as a transcriptional repressor (28, 29). Thus, we used a combination of transcriptional and chromatin assays to determine a possible involvement of GLI proteins in VMP1 activation. Luciferase and expression studies showed that GLI3 can regulate the expression and promoter activity of this molecule in cancer cells. GLI3 is highly expressed in cells carrying a mutant KRASG12D allele (30) and is induced in vivo by oncogenic KRAS. As shown in Fig. 5A, transgenic animals expressing mutant krasG12D showed higher levels of gli3 compared with their wild-type littermates. In MCF10A, 293T, and HeLa cells, overexpression of GLI3 induced autophagy, as shown by the increase of LC3-II (Fig. 5B) and EGFP-LC3 punctae (Fig. 5C). Knockdown of GLI3 led to a decrease in LC3-II in KRAS mutant PANC1 cells (Fig. 5D). Reporter studies demonstrate that overexpression of GLI3 led to an increase in the promoter activity of VMP1 in both KRASG12D-positive PANC1 cells and wild-type HeLa cells (Fig. 5E). This regulatory sequence contains 10 candidate binding sites for GLI transcription factors within the −3000 bp upstream of the transcriptional starting site (Fig. 5F, upper panel). Using a ChIP assay, we confirmed binding of endogenous GLI3 to a region located −1059 bp to −677 bp upstream of the first intron. Fig. 5F (lower panel) shows GLI3 occupancy of this regulatory sequence in PANC1 cells. RNAi targeting GLI3 resulted in a reduction in promoter activity (Fig. 5G) and expression (Fig. 5H) in PANC1 and HeLa cells transfected with KRASG12D in non-transformed cells (MCF10A, HPDE6, and CHO) carrying wild-type copies of this GTPase augmented VMP1 promoter activity (Fig. 3B). Together, these findings define a novel mechanism controlling autophagy in cancer downstream of the oncogene KRAS and identify VMP1 as a target for this oncogenic signaling cascade.

AKT1 Mediates KRAS Induction of VMP1—Analysis of signaling downstream of KRAS demonstrated that an active PI3K-AKT1 pathway is required to maintain the promoter activity and expression of VMP1. Knockdown of AKT kinases (AKT1, AKT2, and AKT3) in PANC1 cells suggested that the regulatory function of this signaling pathway is mediated mainly by AKT1, as RNAi inactivation of this kinase results in a robust reduction of VMP1 expression (Fig. 4A). Overexpression of caPI3K or caAKT1 increased the promoter activity (Fig. 4B) and expression (Fig. 4C) of VMP1, further supporting the role of this pathway in the regulation of VMP1 expression. Finally, we demonstrated that caAKT1 was able to rescue the effect of shRNA targeting the oncogenic KRASG12D in PANC1 cells (Fig. 4D). Indeed, overexpression of caAKT1 restores the activity of the VMP1 promoter in cells transfected with shRNA targeting KRAS. Taken together, these findings suggest the PI3K-AKT1 axis as a mediator of KRAS-induced VMP1 expression.

expression of oncogenic mutant KRASG12D in non-transformed cells (MCF10A, HPDE6, and CHO) carrying wild-type copies of this GTPase augmented VMP1 promoter activity (Fig. 3B). Together, these findings define a novel mechanism controlling autophagy in cancer downstream of the oncogene KRAS and identify VMP1 as a target for this oncogenic signaling cascade.
The results demonstrate that VMP1 is a novel direct target of the GLI3 transcription factor and identify a previously unknown activator function for GLI3.

**KRAS Activates VMP1 by Inducing GLI3-dependent Recruitment of Histone Acetyltransferases**

Transcription factors affect gene expression not only through their inherent activation/repression properties but also through functional interactions with co-regulator molecules. Here, we tested whether activation by GLI3 involved histone acetyltransferases, molecules that have been shown to cooperate with GLI proteins during development (31, 32). RNAi knockdown using two independent VMP1 shRNA constructs or a non-targeting control vector impaired GLI3-mediated activation of the VMP1 promoter in cancer cells (Fig. 6C). Overexpression of GLI3 and p300 led to a synergistic activation of the VMP1 promoter (Fig. 6D). Combined, these data show that the GLI3-p300 activator/co-activator complex is part of the novel signaling pathway controlling the expression and promoter activity of this autophagy-related gene in cancer cells.
GLI3 Acts as Downstream Mediator of KRAS-PI3K-AKT1 Pathway—GLI transcription factors are regulated by Hedgehog and mediate many of its cellular functions during development as well as disease (28). Interestingly, the cancer cell lines used in our studies do not have an active Hedgehog pathway and do not respond to activator ligand in culture (30, 33). These results prompted us to examine whether the KRAS-PI3K-AKT1 pathway can regulate GLI activity in cancer cells. L3.6, PANC1, and MCF10A cells overexpressing a caPI3K (Fig. 7A) or AKT1 (Fig. 7B) showed increased GLI activity, as demonstrated by the induction of a GLI luciferase reporter containing eight consecutive GLI binding sites upstream of the luciferase gene (GLI-luciferase). Conversely, inactivation of this pathway using shRNA targeting oncogenic KRASG12D (Fig. 7C) or a dominant-negative AKT1 (Fig. 7D) demonstrated lower GLI-luciferase activity compared with control transfected cells. Thus, these results provide biochemical evidence supporting a role for the KRAS-PI3K-AKT1 pathway in the modulation of GLI activity in cancer cells.

Next, we sought to determine whether GLI3 was required for the activation of VMP1 by the KRAS-PI3K-AKT1 pathway. PANC1 cells were co-transfected with GLI3 and the constitutively active variants of PI3K and AKT1. The results included in Fig. 8A show that GLI3 is able to synergize with these mutants to activate the VMP1 promoter. Conversely, we found that PANC1 cells co-transfected with a constitutively active AKT1 expression vector as well as shRNA targeting GLI3 or p300, and VMP1 reporter luciferase constructs showed impaired VMP1 promoter activation (Fig. 8B). Similar results were observed in cells co-transfected with p300 shRNA and the KRASG12D oncogenic mutant (Fig. 8C). Finally, overexpression of mutant KRAS increased in the presence of GLI3 and p300 in the VMP1 promoter in HeLa cells (Fig. 8D). Taken together, these results define the PI3K-AKT1-GLI3-p300 pathway as the signaling pathway mediating KRAS induction of the autophagy regulator, VMP1.

DISCUSSION

Autophagy plays a major role in sustaining cellular metabolism and nutrient homeostasis in tumor cells (1–3, 5–7). For instance, autophagy provides cancer cells with a mechanism for escaping unfavorable conditions and surviving nutrient-limiting conditions (4, 5). Numerous signaling pathways have been associated with the initiation and regulation of autophagy in...
cancer cells (1–10, 13). Many of these cascades play a critical role as oncogene or tumor suppressors during tumorigenesis (7). However, the molecular mechanism underlying this phenomenon is still poorly understood. Here, we show that KRAS, an oncogene that induces autophagy, triggers the activation of VMP1 in vitro and in vivo. VMP1, a transmembrane protein essential for the formation of the autophagosome, plays a role in autophagy during stress responses (13–16). Genetic screens in Caenorhabditis elegans identified VMP1 as one of three essential components of the metazoan-specific autophagic pathway (34).

Our study expands on these findings and provides new mechanistic insights into the role of the autophagic process in cancer cells. We define a novel signaling pathway initiated by the oncogene KRAS using the transcription factor GLI3 as an effector to regulate VMP1 expression and autophagy in cancer cells (Fig. 9). Recent studies have demonstrated that KRAS is capable of inducing autophagy in cancer cells through an increase in reactive oxygen species (10, 21–23). The increase in intracellular reactive oxygen species induced by oncogenic KRAS activates JNK and promotes autophagy and the expression of ATG5 and ATG7, which are components of the autophagosome (21). Interestingly, KRASG12D-induced increases in intracellular reactive oxygen species were attenuated by p38 MAPK inhibition, which also suppressed autophagy and subsequent cellular transformation (21).
In the current study, we present data supporting a novel mechanism downstream of KRAS that regulates components of autophagic machinery in cancer cells. This mechanism involves the canonical PI3K-AKT cascade, a novel transcription complex (GLI3-p300), and a new target, VMP1, which is an essential molecule during early stages of the formation of the autophagosome and one that could play a functional role in KRASG12D-induced transformation.

Recent reports by several groups, including ours (18, 30, 32), have demonstrated regulation of the GLI proteins by Hedgehog and non-Hedgehog signaling in multiple cancer types. Here, we provide evidence that the transcription factor GLI3 is a novel effector of the KRAS-PI3K-AKT1 pathway in cancer cells. As mentioned above, GLI3 is a downstream effector of the Hedgehog signaling pathway, a cascade that plays a major role during development (28) as well as carcinogenesis (28, 29). Activation of these transcription factors occurs when the Hedgehog ligand binds to the receptor PATCHED. The binding blocks an inhibitory effect that PATCHED has over SMOOTHENED, a signaling component of the Hedgehog receptor complex, freeing SMOOTHENED to activate GLI transcription factors. These proteins in turn regulate Hedgehog target genes (28).

In this study, we have demonstrated that GLI3 activity is regulated in a SMOOTHENED-independent manner by KRAS-PI3K-AKT1. We have defined GLI3 as a downstream mediator of this signaling cascade and have identified a novel transcriptional activation function for this transcription factor.
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over the autophagy-inducing gene VMP1 in cancer cells. On the basis of our data, we propose a novel pathway that uses GLI3 as the effector for its cellular function and expands the repertoire of signaling cascades that, in addition to Hedgehog, regulate GLI transcriptional activity in tumors.

In summary, our results identify a novel pathway supporting the role of autophagy during carcinogenesis and demonstrate an alternative cascade used by KRAS to induce autophagy in cancer cells. The molecular mechanisms that link oncogenic KRAS to the induction of autophagy and subsequent malignant transformation may provide new insights into the relationship between autophagy and cancer. Lastly, because pharmacological inhibitors for KRAS, PI3K, AKT1, and autophagy (e.g. chloroquine) are being tested in clinical trials for cancer, the biochemical evidence reported here extends the conceptual framework for the better design and interpretation of therapeutic approaches to these dismal diseases.

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