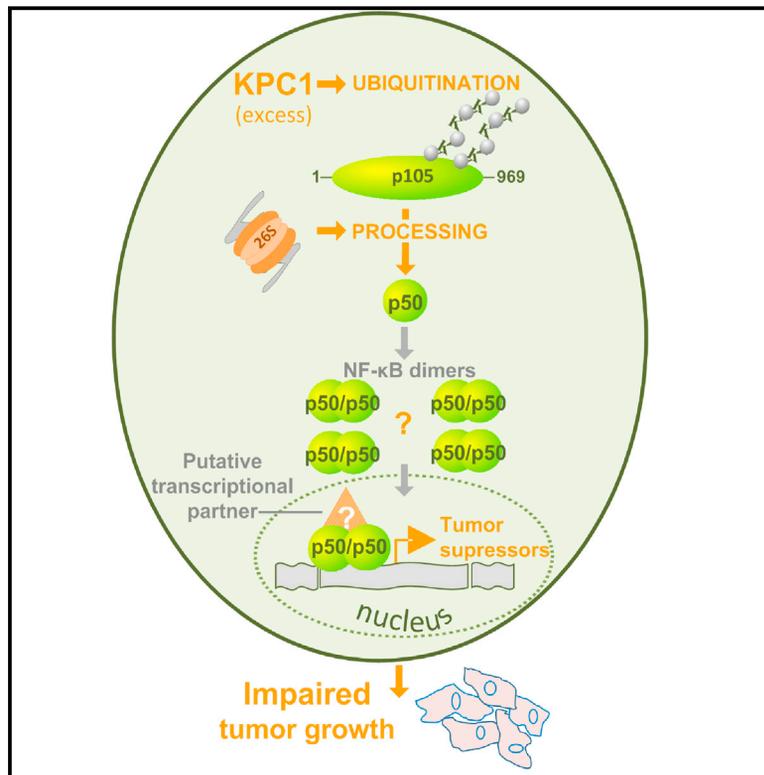


KPC1-Mediated Ubiquitination and Proteasomal Processing of NF- κ B1 p105 to p50 Restricts Tumor Growth

Graphical Abstract



Authors

Yelena Kravtsova-Ivantsiv,
Inna Shomer, ..., Ilana Doweck,
Aaron Ciechanover

Correspondence

aaroncie@tx.technion.ac.il

In Brief

KPC1-dependent ubiquitination of p105 supports limited processing to produce p50, thereby shaping the population of NF- κ B subunits available. Production of p50 appears linked to a tumor-suppressive expression pattern and suggests a balancing of NF- κ B's roles in tumorigenesis.

Highlights

- KPC1 ubiquitinates p105, resulting in its basal and signal-induced cleavage to p50
- KPC1 inhibits tumor growth via regulation of p50-dependent tumor suppressor genes
- KPC1 and p50 levels are correlated and are lower in tumors than in normal tissue
- Excess p50 downregulates p65, possibly lowering tumorigenic levels of p50-p65 NF- κ B

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KPC1-Mediated Ubiquitination and Proteasomal Processing of NF- κ B1 p105 to p50 Restricts Tumor Growth

Yelena Kravtsova-Ivantsiv,^{1,13} Inna Shomer,^{1,13} Victoria Cohen-Kaplan,¹ Berend Snijder,² Giulio Superti-Furga,² Hedva Gonen,¹ Thomas Sommer,³ Tamar Ziv,⁴ Arie Admon,⁴ Inna Naroditsky,⁵ Muhammad Jbara,⁶ Ashraf Brik,^{6,7} Eli Pikarsky,^{8,9} Yong Tae Kwon,^{10,11} Ilana Doweck,¹² and Aaron Ciechanover^{1,10,11,*}

¹The David and Janet Polak Cancer and Vascular Biology Research Center, The Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa 31096, Israel

²CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna 1090, Austria

³Max Delbrück Center for Molecular Medicine, Berlin 13125, Germany

⁴The Smoler Proteomics Center, Faculty of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel

⁵Department of Pathology, Rambam Health Care Campus, Haifa 31096, Israel

⁶Department of Chemistry, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel

⁷The Schulich Faculty of Chemistry, Technion Israel Institute of Technology, Haifa 32000, Israel

⁸Department of Immunology and Cancer Research

⁹Department of Pathology

Institute for Medical Research Israel-Canada (IMRIC), Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

¹⁰Protein Metabolism Medical Research Center

¹¹Department of Biomedical Sciences

College of Medicine, Seoul National University, Seoul 110-799, South Korea

¹²Department of Otolaryngology, Head and Neck Surgery, Carmel Medical Center, Haifa 34367, Israel

¹³Co-first author

*Correspondence: aaroncie@tx.technion.ac.il

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SUMMARY

NF- κ B is a key transcriptional regulator involved in inflammation and cell proliferation, survival, and transformation. Several key steps in its activation are mediated by the ubiquitin (Ub) system. One uncharacterized step is limited proteasomal processing of the NF- κ B1 precursor p105 to the p50 active subunit. Here, we identify KPC1 as the Ub ligase (E3) that binds to the ankyrin repeats domain of p105, ubiquitinates it, and mediates its processing both under basal conditions and following signaling. Overexpression of KPC1 inhibits tumor growth likely mediated via excessive generation of p50. Also, overabundance of p50 downregulates p65, suggesting that a p50-p50 homodimer may modulate transcription in place of the tumorigenic p50-p65. Transcript analysis reveals increased expression of genes associated with tumor-suppressive signals. Overall, KPC1 regulation of NF- κ B1 processing appears to constitute an important balancing step among the stimulatory and inhibitory activities of the transcription factor in cell growth control.

INTRODUCTION

The NF- κ B family of transcription factors is involved in regulation of a variety of genes that control the immune and inflammatory

response, cell survival and death, proliferation, and differentiation. Recently—150 years after Rudolf Virchow discovered the infiltration of tumors with leukocytes and proposed a linkage between chronic inflammation and malignant transformation—it has been shown that the mechanism(s) that underlies this linkage is mediated largely by the NF- κ B family of transcription factors (Ben-Neriah and Karin, 2011; DiDonato et al., 2012). NF- κ B is overexpressed in numerous tumors. It upregulates expression of anti-apoptotic genes such as IAPs, cell-cycle promoters, and growth factors and their receptors (DiDonato et al., 2012). Nevertheless, in some cases NF- κ B was shown to display strong tumor-suppressive characteristics (Perkins, 2012; Pikarsky and Ben-Neriah, 2006). For example, it is involved in regulation of activation-induced apoptosis of T lymphocytes (Ivanov et al., 1997) and in inducing cell-cycle arrest and cell death caused by repression of Bcl2, XIAP, Bcl-X_L, Cyclin D1, and c-Myc that occurs after cell damage. The arrest and death are mediated by p52 dimers (Barré et al., 2010; Barré and Perkins, 2007). Also, it was shown that NF- κ B1^{-/-} cells accumulate alkylator-induced mutations, and NF- κ B1^{-/-} mice develop more lymphomas following alkylating agent-induced DNA damage, again suggesting that NF- κ B1 can act as a tumor suppressor (Voce et al., 2014).

The family members are mostly heterodimers where one of the subunits—p52 or p50—is the product of limited, ubiquitin- and proteasome-mediated processing of a longer (and inactive) precursor, p100 or p105, respectively (Betts and Nabel, 1996; Fan and Maniatis, 1991; Palombella et al., 1994). The other subunit is typically a member of the Rel family of proteins (RelA-p65, RelB, or c-Rel). At times, p50 and p52 can generate homodimers

that cannot act as transcriptional activators since they lack a transactivation domain present in the Rel proteins. In unstimulated cells, the NF- κ B dimers are sequestered in the cytosol attached to ankyrin repeats (ARs) of I κ B inhibitory proteins (I κ B, Bcl3, p100, and p105). A broad array of extracellular signals stimulate degradation of the I κ B proteins, resulting in translocation of the dimers to the nucleus where they initiate different transcriptional programs (Rahman and McFadden, 2011).

Proteasomal processing of p105 occurs under both basal conditions and following stimulation and requires prior ubiquitination (Cohen et al., 2004; MacKichan et al., 1996). One element that was shown to be important in the processing is a long Gly-Ala repeat in the middle of p105 that may serve as a proteasomal “stop signal” (Lin and Ghosh, 1996). In addition to processing, p105 can also undergo complete degradation, releasing NF- κ B dimers anchored to its C-terminal ARs domain. Following stimulation, p105 is phosphorylated on serine residues 927 and 932 by I κ B kinase (IKK β) (Salmerón et al., 2001). This modification recruits the beta-Transducin Repeat Containing Protein (β TrCP) E3 (Orlan et al., 2000), resulting in complete degradation of the molecule (Heissmeyer et al., 2001). The ligase(s) involved in processing of p105 under basal conditions as well as following stimulation has remained elusive.

In the present study, we identified KIP1 ubiquitination-promoting complex (KPC) as the Ub ligase that is involved in both basal and signal-induced processing of p105. KPC is a heterodimer made of KPC1 (*RNF123*) and KPC2 (*UBAC1*). It was shown to degrade the cyclin-dependent kinase inhibitor p27Kip1 in the G1 phase of the cell cycle (Kamura et al., 2004). KPC1 is a RING-finger protein that serves as the ligase. KPC2 interacts with ubiquitinated proteins and with the proteasome via its two Ub-associated domains and a Ub-like domain, acting as a shuttle that promotes the degradation of p27Kip1. It was also shown to stabilize KPC1 (Hara et al., 2005).

RESULTS

Identification of KPC1 as the p105 Ub Ligase

One of the still missing links in the Ub-mediated activation pathway of NF- κ B is the identity of the ligase that ubiquitinates p105, resulting in its proteasomal processing to the p50 active subunit. To identify the ligase, we sequentially fractionated rabbit reticulocyte lysate using different chromatographic principles (Figure 1Ai). Each fraction along the different steps was monitored for E3 activity in a cell-free reconstituted conjugation assay containing *in vitro* translated ³⁵S-labeled p105 as a substrate (Figure 1Aii). To avoid ubiquitination by the β TrCP ligase, we used p105S927A mutant that cannot be phosphorylated by IKK β and therefore cannot bind this E3. Employing mass spectrometric analysis, peptides derived from the KPC Ub ligase were identified in active fractions along the three last chromatographic steps. In the last step of purification (heparin), we identified 58 KPC1 peptides and seven KPC2 peptides covering 43.21% and 19.8% of the open reading frames, respectively (Figure 1B). Because of lack sequence information on rabbit KPC2, we used the sequence of the mouse protein to demonstrate the coverage map. The changes between the two species are negligible (but shown).

To test directly the role of KPC in p105 ubiquitination and processing, we established a cell-free conjugation assay using labeled p105 as a substrate and purified KPC1 or its catalytically inactive species (mutated in the RING domain) KPC1I1256A as the ligase. The wild-type (WT) ligase catalyzed conjugation of p105, whereas the inactive ligase did not (Figure 2A). It appears that KPC1 activity is specific to p105, as it scarcely modifies p100 that is highly homologous to p105 and also undergoes limited proteasomal processing, most probably by a different ligase (Figure S1A).

To demonstrate the ability of KPC1 to modify p105 in cells, we overexpressed Flag-p105 along with HA-Ub in HEK293 cells, in which KPC1 was either silenced (Figure 2B, lane 1), or overexpressed (Figure 2B, lanes 2 and 3). Immunoprecipitation of p105 revealed that it is sparsely ubiquitinated in the absence of the ligase, and ubiquitination is increased significantly following overexpression of KPC1 (Figure 2Bi; immunoprecipitation [IP], compare lanes 1 and 2). Furthermore, we found that p105 binds to KPC1 and co-immunoprecipitates with it (Figure 2Biii; IP, lane 2). In addition, we demonstrated that endogenous KPC1 interacts with endogenous p105 (Figure S1B).

KPC1 Promotes Basal and Signal-Induced Processing of p105

To demonstrate the involvement of KPC1 in p105 processing, we silenced its expression in cells using small interfering RNA (siRNA). As can be seen in Figure 2C, the silencing of KPC1 decreased the amount of p50 generated from p105. In a different experiment, we expressed in HEK293 cells FLAG-p105 along with Myc-KPC1 or Myc-KPC1I1256A. Less p50 was generated in the presence of the KPC1 mutant (Figure S1C).

As noted, processing of p105 occurs also following stimulation. It was interesting to study whether KPC1 can promote p105 processing under these conditions as well. Therefore, we tested the generation of p50 from p105 following expression of constitutively active IKK β (IKK β S176,180E) in the presence (endogenous) or absence (silenced) of KPC1. As expected, the stimulation increased the processing of p105 (compare Figure 2D to Figure 2C; control siRNA). Silencing of KPC1 significantly decreased the generation of p50 following stimulation, strongly suggesting a role for KPC1 in signal-induced processing (Figure 2D). It is known that under the influence of the kinase, the precursor was not only processed but also degraded to a significant extent (compare Figure 2D to Figure 2C and note in particular the decreasing amount of p105 + p50 remained along time following stimulation). It should be noted that the degradation rate of p105 following stimulation was significantly higher in cells that lack KPC1 (Figure 2D). It is possible that the processing of p105 mediated by KPC1 and its degradation mediated by β TrCP occur in parallel. When one process is inactivated, the other becomes dominant. The influence of KPC1 on signal induced-processing of p105 appears to be specific, as its silencing does not affect the processing of p100 following NF- κ B-inducing kinase (NIK) expression (Figure S1D).

In all these experiments, we used exogenously expressed p105. To demonstrate the effect on endogenous p105, we used the human haploid cell line HAP1 in which the single allele of KPC1 or KPC2 were knocked out using the Crispr-CAS

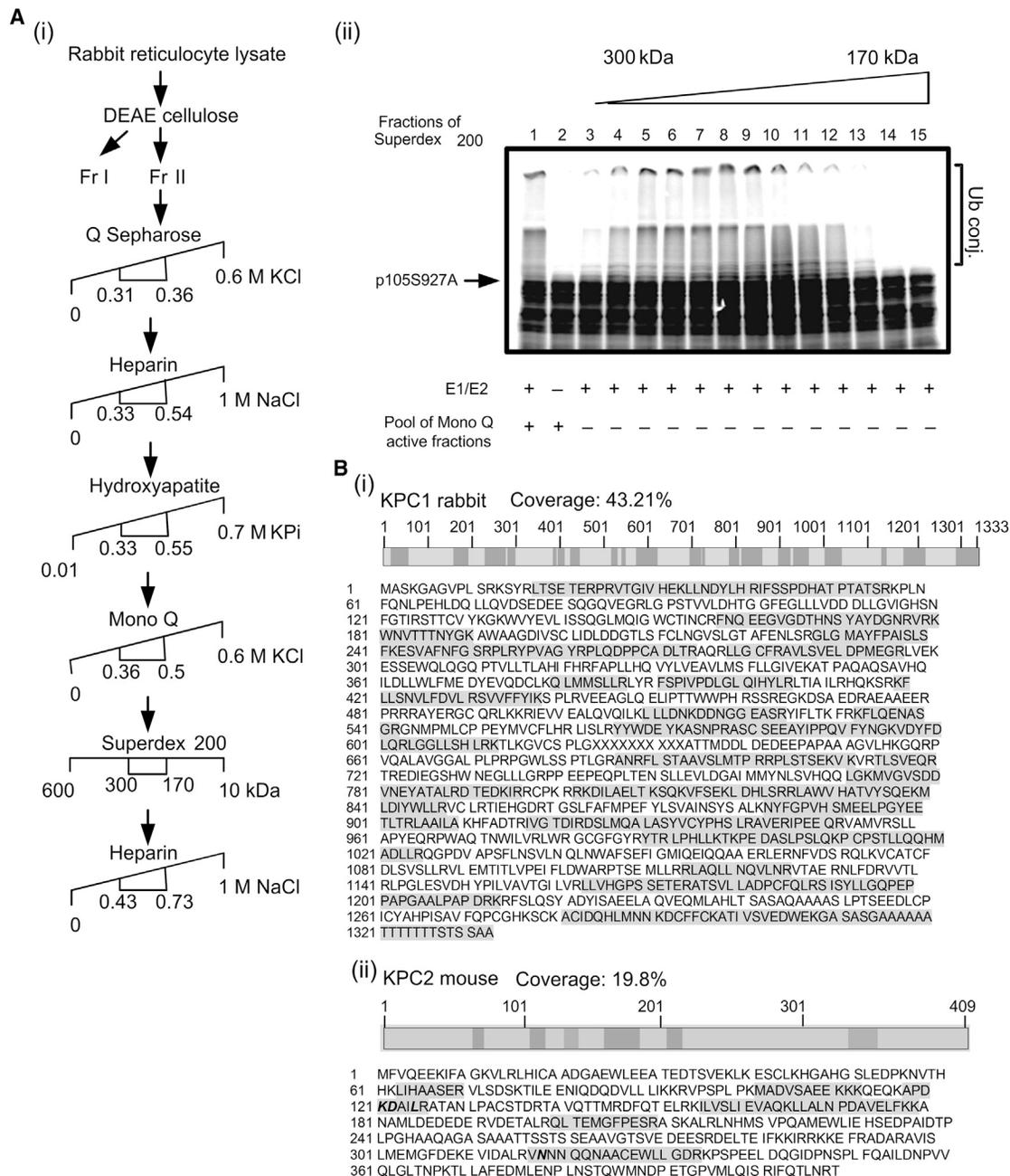


Figure 1. Purification and Identification of the p105 Ub Ligase

(A) (i) Scheme of the chromatographic resolution of Fraction II monitoring the E3 ligating activity toward p105. Numbers represent salt concentrations (M) or molecular weight (kDa) at which the ligating activity was eluted from the respective columns. Fr II, Fraction II. (ii) E3 conjugating activity profile along the fractions resolved by the Superdex 200 gel filtration column. In vitro translated and ^{35}S -labeled p105S927A was ubiquitinated in a reconstituted cell-free system in the presence of the resolved fractions.

(B) (i) Peptide coverage map of rabbit KPC1. The peptides were identified through mass spectrometric analysis of the E3-containing fractions resolved by the last, Heparin-based column. (ii) Peptide coverage map of mouse KPC2. The peptides were identified through mass spectrometric analysis of the E3-containing fractions resolved by the last, Heparin-based column. Residues marked in bold and italics denote differences in sequence between mouse and rabbit.

technology. Elimination of KPC1 or KPC2 (that stabilizes KPC1 [Hara et al., 2005]; note that removal of KPC2 results in a significant decrease in the level of KPC1; Figure 2E) decreased the generation of p50 both in the presence or absence of TNF α

(Figure 2E). In contrast, the level of p65 was not affected. The finding that p50 is still present, albeit in a decreased level, in the KPC1 KO cells, may be due to the activity of another, yet to be identified ligase, and/or to co-translational processing of

the nascent peptide that occurs before completion of the p105 precursor synthesis (Lin et al., 1998). It should also be noted that the effect of KPC1 on p50 generation is significantly more pronounced in tumors growing in mice than in cultured cells (see below).

Our finding that KPC1 mediates processing under both basal and stimulated conditions prompted us to dissect the mechanism involved. We monitored the interaction between KPC1 and p105 under basal and stimulated conditions and found that expression of constitutively active IKK β results in increased interaction between the two as assayed by co-immunoprecipitation (Figures 2F and S1E). The finding that the interaction of p105S927A with KPC1 is not affected by IKK β (Figure 2Fi, lanes 4 and 5) attests to the specificity of the effect of IKK β in phosphorylating a specific Ser residue (927) in p105. As expected, we found that ubiquitination of phosphorylated p105 by KPC1 is stronger compared to that of the non-phosphorylated species (Figure S1F).

To further confirm that KPC1 interacts more efficiently with phosphorylated p105, we designed an experiment in which we competed on the binding of p105 to the ligase with a synthetic phosphorylated peptide derived from the p105 IKK β -phosphorylation site. The phosphorylated peptide inhibited ubiquitination of p105 by KPC1 to a larger extent compared with its

non-phosphorylated species, both in a crude system and in a system made of purified components (Figures 2G and S1G, respectively).

Role of KPC2 in KPC1-Mediated p105 Ubiquitination and Processing

At that point, it was important to study the role of KPC2, the partner of KPC1 in the heterodimeric ligase complex, in p105 modification and processing. We noted that its addition to a reconstituted cell-free system decreases significantly the ubiquitination of p105 by KPC1 (Figure 2H). This was true also when p105 was purified by a specific antibody, ruling out a possible effect of other components present in the mixture in which the labeled p105 was translated (Figure S2Ai). To rule out that the reduced ubiquitination of p105 in the presence of KPC2 is due to a possible deubiquitinating activity of the protein, we added it to the cell-free ubiquitination system after KPC1, when most of the ubiquitination reaction was completed. It had no effect on the conjugates pattern (Figure S2Aii). The interference of KPC2 in chain formation appears to be specific to KPC1 and p105, as it did not affect the ligase activity of E6-AP toward RING1B^{53S} (Zaaroor-Regev et al., 2010) (Figure S2B).

Importantly, in correlation with the suppressive effect of KPC2 on KPC1-mediated ubiquitination of p105, silencing of KPC2

Figure 2. p105 Is a Substrate of KPC1 in a Cell-free System and in Cells, Both under Basal Conditions and following Signaling

(A) Ubiquitination of in vitro translated and ³⁵S-labeled p105 by Fraction II and purified KPC1-FLAG-TEV-6xHIS or KPC111256A-FLAG-TEV-6xHIS in a reconstituted cell-free system. Fr II, Fraction II.

(B) KPC1 ubiquitinates p105 in cells. HEK293 cells that were transfected with siRNA to silence KPC1 (lane 1) or with control siRNA (lanes 2 and 3), were also transfected with cDNAs coding for FLAG-p105 (lanes 1 and 2), HA-Ub (lanes 1–3), and Myc-KPC1 (lanes 2 and 3). FLAG-p105 and its conjugates were immunoprecipitated from the cell lysates using immobilized anti-FLAG (IP; lanes 1–3), resolved via SDS-PAGE, and visualized using anti-HA (Bi) or anti-FLAG (Bii). KPC1 was visualized using a specific antibody to the protein (Biii). Ten percent of total cell lysates (TCL; lanes 1–3) were analyzed for expression of FLAG-p105, HA-Ub or Myc-KPC1, using anti-HA (Bi), anti-FLAG (Bii), or anti-KPC1 (Biii), respectively. IP, immunoprecipitation; WB, western blot.

(C) Silencing of KPC1 affects basal processing of p105. HEK293 cells were transfected with control siRNA (lanes 1–3) or siRNA to silence KPC1 (lanes 4–6). After 24 hr, cells were transfected with cDNAs coding for FLAG-p105. Processing of p105 was calculated as the ratio between the amount p50 at the specified time and the sum of p50 + p105 at time zero (in order to disregard degradation of p105 in our calculations), multiplied by 100%. The amount of p50 + p105 remained (reflecting degradation along time) was calculated as the sum of p50 + p105 measured at the relevant time point, divided by the sum of p50 + p105 at time zero, multiplied by 100%.

(D) Silencing of KPC1 inhibits signal-induced processing of p105. HEK293 cells were transfected with control siRNA (lanes 1–3) or siRNA that targets KPC1 (lanes 4–6). After 24 hr, cells were transfected with cDNAs coding for FLAG-p105 and IKK β S176,180E. Twenty-four hours after transfection (in the experiments depicted under C and D), cycloheximide was added for the indicated times, and cells were lysed, resolved via SDS-PAGE, and proteins visualized using anti-FLAG, anti-KPC1 or anti-actin. Processing and degradation were assessed as described under (C). Chx, cyclohexamide. Actin was used to ascertain equal protein loading.

(E) Deletion of KPC1 or KPC2 genes inhibits basal and TNF α -induced processing of endogenous p105. Lysates were prepared from HAP1 control or HAP1 cells knocked out for the genes coding for KPC1 or KPC2. The lysates were resolved via SDS-PAGE, and proteins were visualized using anti-NF- κ B1, anti-KPC1, anti-KPC2, anti-p65, or anti-actin. The amount of p105 processed was calculated as the ratio between the generated p50 and the sum of p50 + p105, multiplied by 100%.

(F) The interaction between p105 and KPC1 increases following signaling. HEK293 cells were transfected with cDNAs coding for FLAG-p105 (lanes 2 and 3) or FLAG-p105S927A (lanes 4 and 5) along with Myc-KPC1 (lanes 1–5) and FLAG-IKK β (lanes 2 and 4) or FLAG-IKK β S176,180E (lanes 3 and 5). FLAG-p105 and FLAG-p105S927A were immunoprecipitated from the cell lysate using immobilized anti-FLAG (lanes 1–5), and the bound KPC1 was visualized with anti-KPC1 (Fi). Immunoprecipitated p105s were visualized using anti-FLAG (Fii).

(G) A phosphorylated peptide corresponding to the signaled sequence in p105 inhibits its ubiquitination. In vitro translated and ³⁵S-labeled p105 was ubiquitinated by purified KPC1-FLAG-TEV-6xHIS (lanes 2–9) in a reconstituted cell-free system in the presence of a phosphorylated peptide derived from the signaled sequence of p105 (lanes 6–8), or in the presence of its non-phosphorylated counterpart (lanes 3–5). Presented is the change (in %) of unconjugated p105 remained following addition of increasing concentrations of the peptides (compared to a system to which a peptide was not added; lane 2).

(H) KPC2 attenuates ubiquitination of p105 by KPC1. Ubiquitination of in vitro translated and ³⁵S-labeled p105 by purified KPC1-FLAG-TEV-6xHIS in the presence or absence of HIS-KPC2 was carried out in a cell-free reconstituted system.

(I) KPC2 attenuates processing of p105 in cells. HEK293 cells were transfected with control siRNA (lanes 1–3) or siRNA to silence KPC2 (lanes 4–6). After 24 hr, cells were transfected with cDNAs coding for FLAG-p105 and generation of p50 was monitored 24 hr later. Processing of p105 was calculated as described under (C).

(J) KPC1 modifies lysine residues in the C-terminal segment of p105. In vitro-translated and ³⁵S-labeled WT and the indicated p105 mutants were subjected to ubiquitination by purified KPC1-FLAG-TEV-6xHIS in a reconstituted cell-free system.

See also Figures S1 and S2.

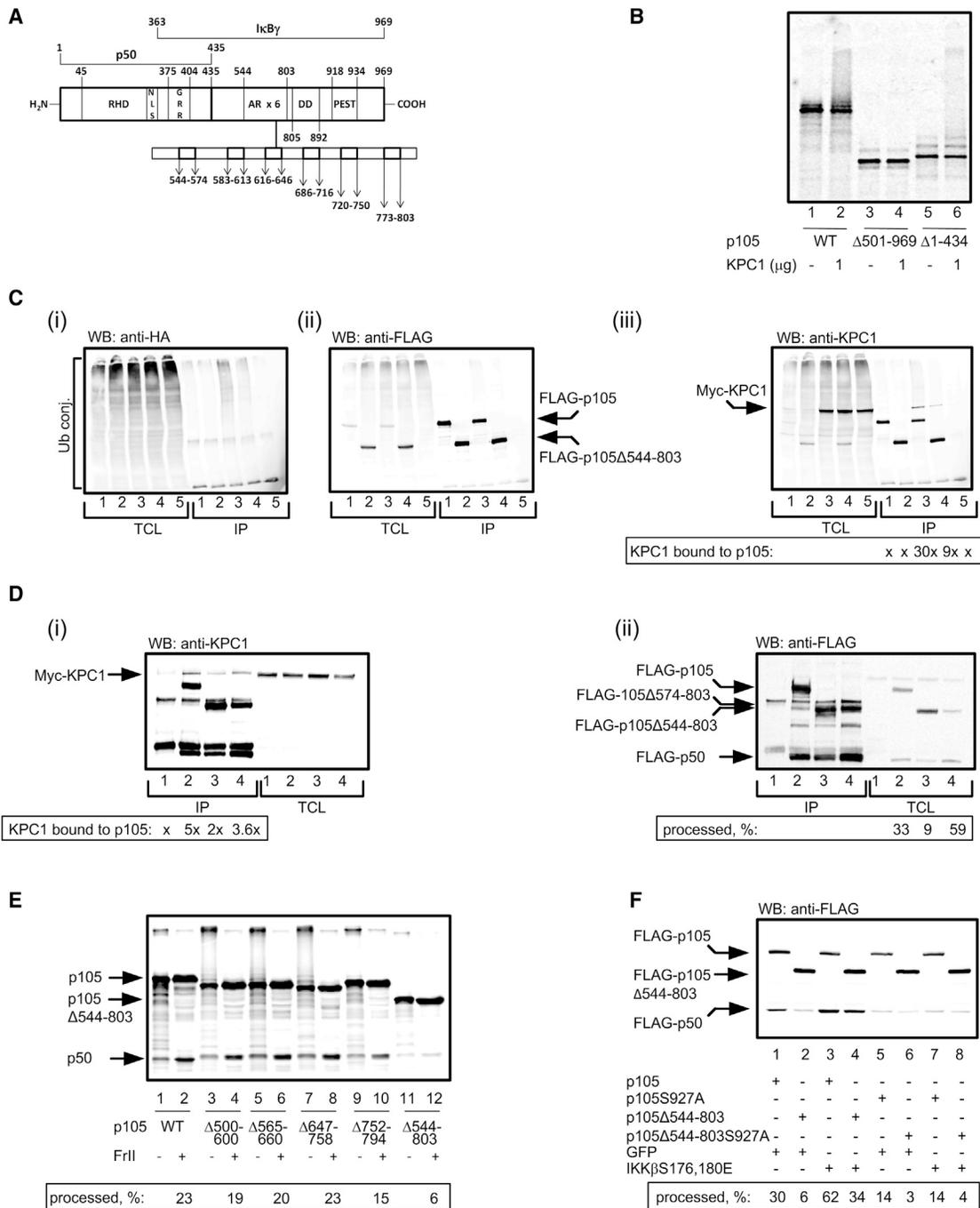


Figure 3. KPC1-Dependent Ubiquitination and Processing of p105 Require the ARs of p105

(A) Schematic representation of p105 domains. Numbers denote the respective residue along the protein sequence. RHD, Rel homology domain; NLS, nuclear localization signal; GRR, glycine rich repeat; AR, ankyrin repeats (all six of them are marked).

(B) The ARs-containing C-terminal half of p105 is ubiquitinated by KPC1. In vitro-translated and ³⁵S-labeled p105, p105Δ501-969 or p105Δ1-434 were subjected to ubiquitination by purified KPC1-FLAG-TEV-6xHIS in a reconstituted cell-free system.

(C) The ARs of p105 are essential for binding of KPC1 and for its ubiquitination by the ligase in cells. HEK293 cells that were transfected with siRNA to silence KPC1 (lanes 1 and 2) or with control siRNA (lanes 3–5), were also transfected with cDNAs coding for FLAG-p105 (lanes 1 and 3), p105Δ544-803 (lanes 2 and 4), HA-Ub (lanes 1–5), and Myc-KPC1 (lanes 3–5). The different FLAG-p105 species and their conjugates were immunoprecipitated from the cell lysates by immobilized anti-FLAG (IP; lanes 1–5).

(D) KPC1 interacts with a single AR in p105. HEK293 cells were transfected with cDNAs coding for FLAG-p105 (lane 2), FLAG-p105Δ544-803 (lane 3), or FLAG-p105Δ574-803 (lane 4), along with Myc-KPC1 (lanes 1–4). The different FLAG-p105 species were immunoprecipitated from the cell lysates using immobilized anti-FLAG (IP; lanes 1–4).

(legend continued on next page)

increased the formation of p50 (Figure 2I). That, despite the fact that the short-term silencing reduced partially the level of KPC1 via its effect (or absence thereof) on the stabilization of the ligase (Figure 2I; note the change in the level of KPC1 following KPC2 silencing).

Identification of the Ub Anchoring Sites on p105 Modified by KPC1

We have already shown that multiple lysines in the C-terminal segment of p105 are required for its ubiquitination and processing (Cohen et al., 2004; Kravtsova-Ivantsiv et al., 2009) in crude extracts. It was therefore important to show that this is true also for KPC1. Progressive removal of all lysine residues from the C-terminal segment (Figure S2C) resulted in corollary decrease in conjugation of p105 by KPC1 in a cell-free assay (Figure 2J) and in processing of the precursor in cells (Figure S2D).

The C-Terminal ARs of p105 Are Necessary for Its Interaction with KPC1 and for Its Subsequent Ubiquitination and Processing

p105 harbors several domains: REL homology domain (RHD), nuclear localization signal (NLS), and a glycine rich repeat (GRR) in its N-terminal segment, and ARs, death domain (DD), and a PEST (proline, glutamate, serine, and threonine) sequence in the C-terminal segment (Figure 3A). We examined which of these domains is necessary for ubiquitination by KPC1. As can be seen in Figure 3B, removal of the C-terminal segment abolished altogether conjugation in a cell-free system, whereas removal of the N-terminal segment had no effect. Subsequently we found that removal of all six ARs (p105 Δ 544-803) affected significantly the ubiquitination of p105 by KPC1 (Figure S3A, compare lanes 2 and 12). Partial deletion of the repeats affected conjugation only slightly (compare lane 2 to lanes 4, 6, 8, and 10).

Similar results were obtained in experiments carried out in cells. Overexpression of KPC1 increased the ubiquitination of WT p105, but much less so of p105 that lacks all its ARs (Figure 3Ci; IP, compare lane 4 to lane 3). Importantly, in parallel, we observed also a decrease in the interaction between the ARs'-truncated p105 and its ligase compared to WT p105 (Figure 3Ciii; IP, compare lanes 4 and 3).

To rule out the possibility that the decrease in ubiquitination of p105 that lacks all its ARs is due to removal of the eight lysine residues in the repeats, we generated a mutant p105 in which all these lysines were substituted by arginines. The ubiquitination of the K to R mutant as well as its interaction with KPC1, were similar to that of WT p105 (Figures S3Bi and ii, respectively). An interesting question relates to the number of ARs necessary for ubiquitination and processing of p105. We constructed a

p105 mutant where all ARs except one have been deleted (p105 Δ 574-803). The single remaining AR was sufficient to bind KPC1 and to promote processing similar to that observed for WT p105 (Figure 3D). Thus, it appears that the ARs are redundant with relation to binding of KPC1.

Last, it was important to demonstrate whether the ARs-dependent ubiquitination increases the processing of p105. As can be seen in Figure 3E, mutant p105 that lacks all ARs, is processed much less efficiently compared to the WT species and to one lacking only some of the repeats (compare lane 12 to lanes 2, 4, 6, 8, and 10). A similar result was obtained also in cells (Figure 3F, lanes 1 and 2). Mutant p105 in which all lysine residues in the ARs were substituted with arginines (FLAG-p105K8R), is processed similarly to WT p105 (Figure S3C, lane 3), strongly suggesting that the ARs are required for the binding, ubiquitination, and processing of p105, but do not serve as ubiquitination sites essential for processing.

It appears that the ARs are also involved in signal-induced processing of p105, as their removal significantly decreased IKK β -mediated generation of p50 (Figure 3F, compare lane 4 to lane 3). As expected, FLAG-p105S927A and FLAG-p105S927A Δ 544-803 did not respond to IKK β -mediated phosphorylation (Figure 3F, lanes 7 and 8).

Overexpression of KPC1 or p50 Suppresses Tumor Growth

Since NF- κ B dimers are known to affect cell survival, proliferation, and tumor progression, it was interesting to study the outcome of KPC1 on cell growth. Initially, we monitored the influence of overexpressed KPC1 on anchorage-independent growth in MB-MDA 231, U2OS, and U87-MG cells, and found that it inhibits colony formation by 36%, 32%, and 52%, respectively, compared to controls (Figures 4A–4C). Importantly, this effect was abrogated in cells overexpressing the inactive ligase species KPC1I1256A, suggesting that the inhibitory effect is due to the ligase activity (Figure 4C). Cells expressing p50 showed an even stronger inhibition of colony formation (73% for both MB-MDA 231 and U87-MG cells; Figures 4A and 4C), strongly suggesting that the effect of the ligase is mediated through its activity on p105, resulting in excessive generation of p50. Supporting the linkage is the finding that silencing of p105 abrogated the strong suppressive effect of KPC1: the number of colonies formed using cells that overexpress KPC1 in the absence of p105 was 7.5-fold larger than that formed in its presence (Figure 4D). It was important to study whether the growth suppressive effect of KPC1 and p50 is not due to induction of apoptosis. Thus, we stained U87-MG cells that overexpress these proteins for cleaved caspase 3. As can be seen in Figure S4, we could not detect the apoptotic marker. For that

(E) p105 that lacks its ARs is processed less efficiently in a cell-free system. The different ³⁵S-labeled p105 species were processed in a cell-free reconstituted system in the presence or absence of Fraction II as indicated. Fr II, Fraction II.

(F) Deletion of the ARs of p105 affects both its basal and signal-induced processing. HEK293 cells were transfected with cDNAs coding for FLAG-p105, FLAG-p105 Δ 544-803, FLAG-p105S927A, or FLAG-p105S927A Δ 544-803 along with either GFP or IKK β as indicated.

In (C), (D), and (F), proteins were resolved via SDS-PAGE, blotted onto nitrocellulose membrane, and p105 and p50 were detected using anti-FLAG, KPC1 was detected using anti-KPC1, and Ub conjugates were detected using anti-HA. Ten percent of the total cell lysates (TCL) were analyzed for the expression of proteins. The SDS-PAGE-resolved labeled proteins in the experiments shown in (B) and (E) were visualized using PhosphorImaging. Processing was assessed as described under Figure 2E.

See also Figure S3.

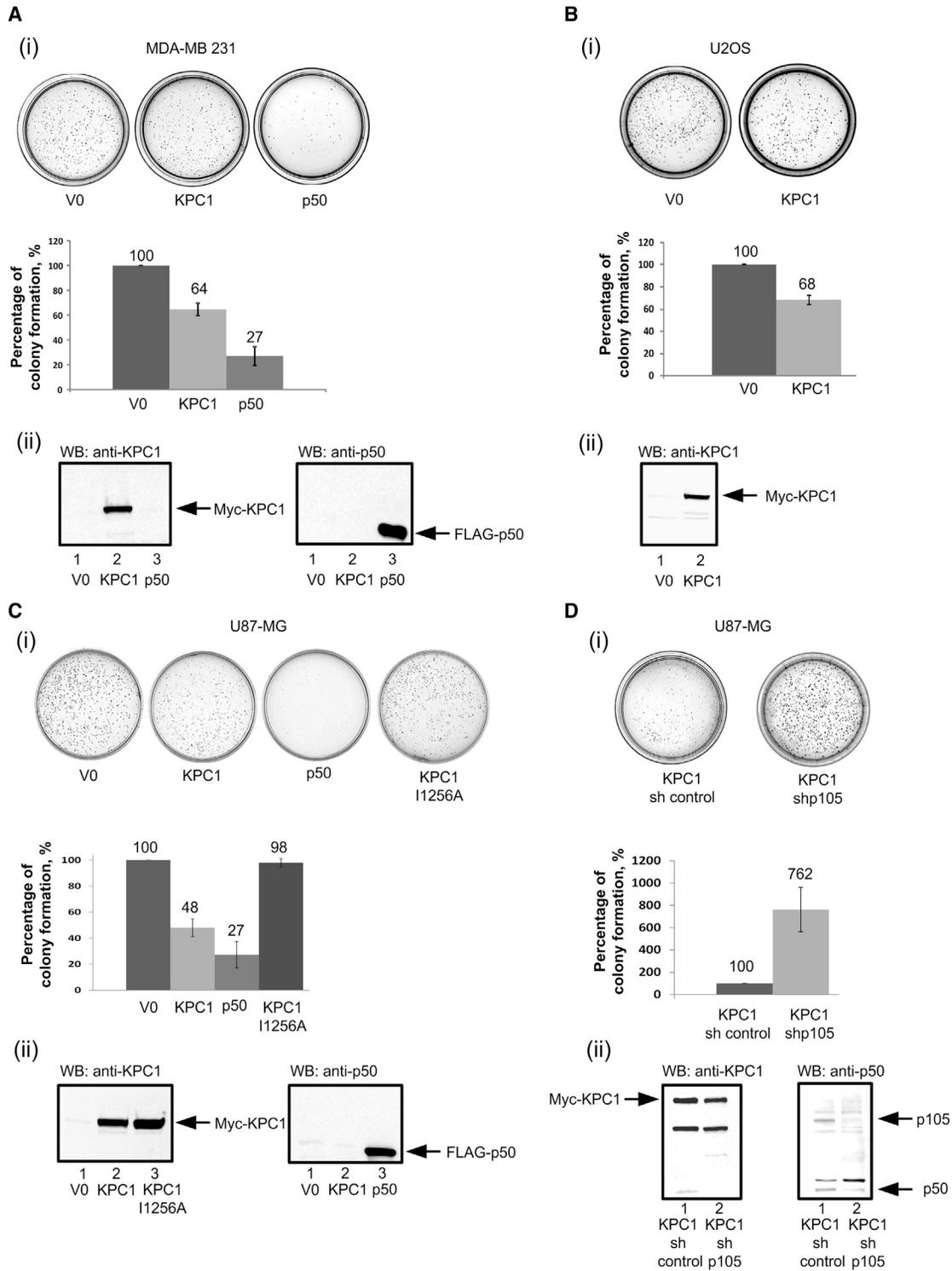


Figure 4. KPC1 and p50 Suppress Anchorage-Independent Growth of Cells

Suppression of colony formation by overexpressed KPC1 or p50 in MDA-MB 231 (Ai), U2OS (Bi), and U87-MG (Ci and Di) cells. Cells were stably transfected with V0, or with cDNAs that code for Myc-KPC1, Myc-KPC1I1256A or FLAG-p50, or with cDNA coding for Myc-KPC1 along with control shRNA or shRNA to silence p105, as indicated, and were seeded on soft agar plates. After 3 weeks, the colonies were stained with 0.05% crystal violet. Data derived from five experiments (\pm SEM) are presented graphically. Expression of KPC1, KPC1I1256A, p50, and p105 is shown in (Aii), (Bii), (Cii), and (Dii). See also Figure S4.

experiment, it was also important to demonstrate that the suppressive effect of KPC1 and p50 is not due to some non-specific effect of the overexpression of the proteins. The unleashing of growth in the presence of overexpressed KPC1 but in the absence of p105 (Figure 4D), strongly suggests that the effect of KPC1 is indeed specific.

These observations prompted us to study the effect of KPC1 in a tumor model in mice. We generated xenografts stably overexpressing V0, KPC1, KPC111256A, or p50. Both the growth rate and weights of tumors expressing KPC1 and p50 were significantly smaller compared to those that harbor V0 or KPC111256A (Figures 5Ai and 5Aiii for xenografts derived from U87-MG cells, and Figure 5Aii for xenografts derived from MDA-MB 231 cells). Importantly, in tumors that overexpress KPC1, the level of p50 is significantly higher compared with tumors that express V0 (Figure 5Aiv), suggesting again a direct linkage between the KPC1 ligase activity and increased generation of p50. Interestingly, in tumors that overexpress KPC1 or p50, we also observed a significant decrease in the level of p65 (Figure 5Aiv). This finding raises the possibility that a different NF- κ B transcription factor is generated under the influence of KPC1, possibly a p50 homodimer (see Discussion). To demonstrate that there are indeed changes in NF- κ B species in human tumor xenografts overexpressing KPC1 and p50, we used electrophoretic mobility shift assay (EMSA) to monitor the activity of the transcription factor. As can be seen in Figure S5A, there is a significant decrease in the ability of “canonical” NF- κ B to bind its consensus DNA sequence following overexpression of KPC1 and even more so following overexpression of p50.

Of note, all the effects on tumor growth (reduction in colony formation, tumor growth rate, and weight) were more prominent in p50-expressing tumors than in their KPC1-overexpressing counterparts. This is not surprising, as p50 is the product of KPC1 activity, and its direct expression has a stronger effect.

The functional linkage between KPC1 and p50 can also be observed in staining of specific proliferation and differentiation markers in the mice-derived tumors. The overexpression of KPC1, but not of KPC111256A, results in increased appearance of nuclear NF- κ B (Figure 5B), a significant decrease in the proliferation marker ki-67, and an increase in the glial fibrillary acidic protein (GFAP), a known glial cells differentiation marker. Suspecting that KPC1 stimulates apoptosis, we looked for an increase in cleaved caspase 3, however, there was no change in the levels of the active enzyme compared to control sections. Staining of p27Kip1—a suggested substrate of KPC1 (Kamura et al., 2004)—did not show any change in the protein level (Figure 5B). This may be due to the differences in the systems studied.

KPC1 Regulates Expression of a Subset of p50 Target Genes

We next analyzed the profile of gene expression in the tumors using RNA sequencing (RNA-seq) analysis of transcripts mapped to the human genome (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60530>; Table S1). The altered gene expression patterns revealed a strong similarity between overexpression of p50 and KPC1 in U87-MG xenografts (correlation of 0.51, p value $< 10^{-300}$; Figure 6Ai), with 48 downregulated

and 534 upregulated genes that were consistent and significant in all replicates (Figure 6Aii; Table S2). The relative transcript levels of selected genes that were shown to be significantly upregulated and downregulated in RNA-seq analyses, was corroborated by quantitative real-time PCR (qRT-PCR) (Figure S5B). Functional analysis revealed highly significant enrichment in glycosylated and extracellular matrix proteins, and upregulation of genes expressing proteins involved in cell-cell and cell-substrate adhesion, regulation of cell migration, cell junctions, vasculature development, wound healing, and cell-cell signaling (Figure 6Aiii), suggesting a re-establishment of “social” micro-environmental interactions in the p50- and KPC1-overexpressing glioblastoma tumors (Bonavia et al., 2011). Downregulated processes included a reduced response to hypoxia required for maintaining glioblastoma stem cells (Heddleston et al., 2009), as well as reduced positive regulation of cell migration (Figure 6Aiii). Of the consistently changed genes, 21 are known NF- κ B targets (p value $< 3.4 \times 10^{-9}$; <http://www.bu.edu/nf-kb/gene-resources/target-genes/>). To further assess if the observed reduction in tumor size was the consequence of a reduction in proto-oncogenes and/or of an increased expression of tumor suppressor genes, we gathered gene annotations from various sources. Enrichment analysis on these gene annotations revealed a significant (p value $< 1.4 \times 10^{-18}$) increase in the expression of 40 tumor suppressor genes, with no significant change in other classes (Figure 6B).

Finally, we integrated functional annotation enrichment and protein-protein interactions for the differentially regulated genes, which revealed a dense network of upregulated genes revolved around a few downregulated ones, such as interleukin-6 (IL-6), interleukin-6 receptor (IL-6R), and vascular endothelial growth factor A (VEGFA) (Figure 6C; Data S1). We included KPC1 and NF- κ B in this analysis to retrieve possible known interactions, although KPC1 had no known interactions with any of the differentially regulated genes. Closer look at the core interaction network (Figure 6C, inset magnification) that included NF- κ B is most prominently annotated with “regulation of cell migration” genes. Most other core network genes are upregulated and include many well-known tumor-suppressor genes.

Taken together, our findings strongly suggest a model of KPC1/p50 driven glioblastoma tumor growth inhibition, that centers around downregulated high mobility group protein HMGI-C (HMGA2), lin-28 homolog A (LIN28), IL-6/IL-6R, and VEGFA, and upregulated tumor suppressors, which in combination control the tumor-microenvironment as well as glioblastoma stem cell maintenance.

Correlation between Expression of KPC1 and p50 in Human Tumoral and Normal Tissues

Finally, we examined the relationship between KPC1 and p50 in human tumors and normal tissues. Immunohistochemical staining of the two proteins (the two antibodies were shown to be specific; see Figure S6) revealed a high correlation between them in squamous cell carcinoma of head and neck (SCCHN, 52 sections; p value < 0.005 ; Figure 7Aii), breast cancer (105 sections; p value < 0.0001), and glioblastoma (192 sections; p value < 0.0017) (Figure 7Ai). It should be emphasized though

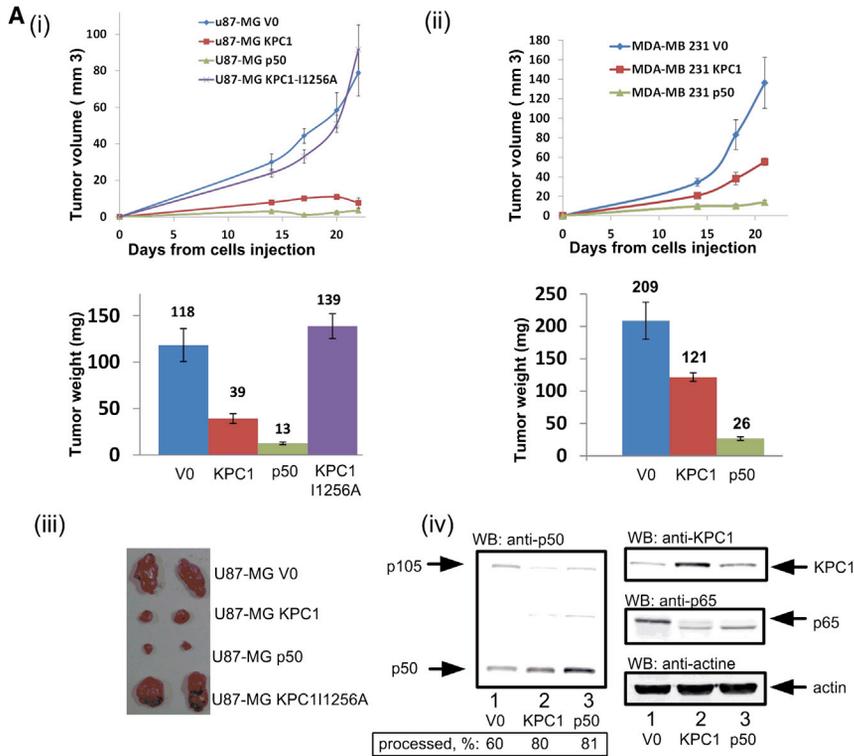


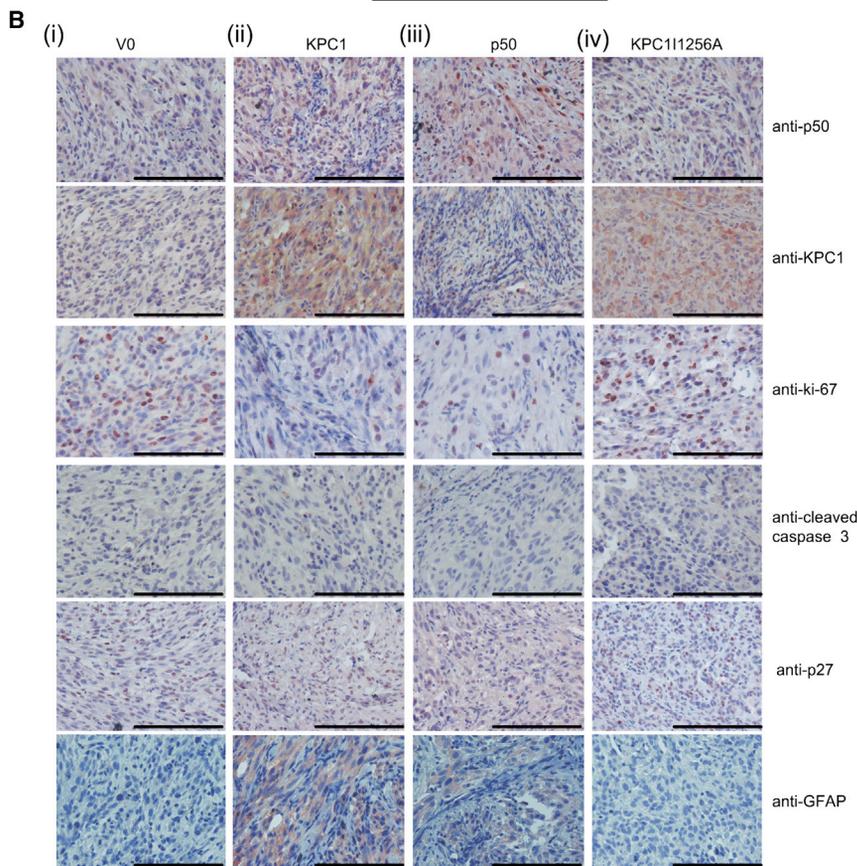
Figure 5. KPC1-Mediated Excessive Generation of p50 Inhibits Tumor Growth

(A) Growth rates and weights of tumor xenografts grown in mice, and derived from U87-MG (i) and MDA-MB 231 (ii) cells expressing V0, Myc-KPC1, and FLAG-p50. Data represent the mean of seven xenografts \pm SEM (iii) Tumors derived from U87-MG cells 3 weeks after inoculation. (iv) Enhanced generation of p50 and disappearance of p65 in tumors that overexpress KPC1 and p50. Proteins were resolved via SDS-PAGE, blotted onto nitrocellulose membrane, and detected using the appropriate antibodies. Processing was assessed as described under [Figure 2E](#).

(B) Immunohistochemical staining of p50, KPC1, ki-67, cleaved caspase 3, p27Kip1, and GFAP in xenografts of U87-MG cells stably expressing V0 (i), Myc-KPC1 (ii), FLAG-p50 (iii), or KPC1 I1256A (iv).

All scale bars, 100 μ m. Tumors were grown in mice and stained as described in the [Experimental Procedures](#).

See also [Figure S5](#).



that the linkage may be tumor-specific and not common to all patients with the “same” tumor.

To test the hypothesis that loss of KPC1 and nuclear p50 can be involved in the pathogenesis of malignant transformation, we analyzed the staining of the two proteins in SCCHN, breast cancer, and glioblastoma, and compared it to their staining in the normal parallel tissue. We observed a strong decrease in tumor samples stained for nuclear p50 compared to the healthy tissue (Figure 7B). As for KPC1, we observed a significant decrease in staining intensity (reflecting the amount of the protein) in cancerous compared to normal tissue in both SCCHN and glial cells, but not in breast cancer. Also, we noted a significant decrease in the number of tumor samples stained for KPC1 in SCCHN (Figure 7B). Taken together, these findings suggest that nuclear p50 is indeed a tumor suppressor lost in many malignancies. At least part of this p50 loss may be due to loss of KPC1 which catalyzes its formation, though this may not be common to all tumors.

DISCUSSION

The vast majority of substrates of the Ub proteasome system are completely degraded. One intriguing and exceptional case is that of the p105 precursor of NF- κ B that can be either completely degraded or processed in a limited manner to yield the p50 active subunit of the transcription factor. The “decision-making” mechanism resulting in one of the two distinct processes has remained largely elusive. The β TrCP Ub ligase has been identified as the tagging enzyme involved in complete degradation of p105, whereas the ligase involved in processing has remained unknown. We have now identified the KPC complex as the putative p105-processing ligase (Figures 1, 2, and 3).

Now that the two E3s involved in degradation and processing of p105 have been identified, it is still not known why ubiquitination by one enzyme results in a completely different fate of p105 than ubiquitination by the other and what determines the timing of the two reactions. It is possible that the two ligases catalyze the formation of chains that differ in their anchoring sites, length, and/or internal linkages. These, in turn, affect the recognition and mechanism of action of the 26S proteasome. As for timing, it can be that different physiological conditions and/or the degree of saturation of the ARs with bound p50s are involved in the “decision-making” process of whether the molecule will be processed or destroyed completely.

Studying the biological implications of manipulating KPC1 revealed that it suppresses anchorage-independent growth in a manner that is dependent on its ligase activity and the presence of p105. A corollary strong tumor-suppressive effect was demonstrated in xenografts of human tumors (Figures 4, 5, and

6). This effect is caused probably by a significant increase in an entire set of tumor suppressors, some of them like the brain-specific protein cell adhesion molecule 3 (CADM3) (Gao et al., 2009), was found inactivated in glioblastoma.

An important question relates to the transcriptional mechanism by which KPC1 and p50 exert their tumor-suppressive effect. An obvious assumption is that the stoichiometric excess of p50 generated by KPC1 would generate mostly p50-p50 homodimers rather than the “canonical” tumorigenic p50-p65 heterodimers. In line with this finding is also the observation that p65 level is decreased in KPC1-overexpressing as well as in p50-overexpressing xenografts (Figure 5Aiv). It appears that each dimer of NF- κ B family has unique and even opposing biological function(s) and regulates a distinct subset of downstream genes (Siggers et al., 2012). p50 homodimer is supposed to act as a transcriptional repressor because it does not contain a transactivation domain (May and Ghosh, 1997). However, studies in vitro have shown that p50 homodimer can interact with different transcriptional modulators, such as Bcl-3 (Fujita et al., 1993), p300 (Deng and Wu, 2003), or HMGA1/2 (Perrella et al., 1999) that are involved in chromatin remodeling. Disproportionate p50 may shift the composition of NF- κ B dimers, resulting in overall tumor-suppressive effect. Indeed, following overexpression of KPC1 or p50, there is a decrease in the level of what is probably the “canonical” tumorigenic NF- κ B (p50-p65; Figure S5A).

Importantly, we found a strong correlation between the expression of KPC1 and that of p50 in human tumors (Figure 7A). Moreover, we found a significant decrease in nuclear p50 and KPC1 staining intensity in tumors compared to non-malignant tissue (Figure 7B). This observation suggests that loss of nuclear p50 may trigger malignant transformation. In line with these findings are data collected in the Catalog Of Somatic Mutations in Cancer (COSMIC) that show a significantly greater number of common tumors (e.g., breast, lung, CNS, and uterine cervix) with decreased expression of KPC1 transcripts compared to those with high expression (<http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=RNF123&ln1=RNF123&start=1&end=1315&coords=AA%3AAA&sn=&ss=&hn=&sh=&samps=1001&expn=over&expn=under&id=4185>).

EXPERIMENTAL PROCEDURES

Materials, Plasmids, Expressed Proteins, and Cells

All materials (including plasmids and their construction, expression of proteins and their purification, and cultured cells and their manipulation), are described in the [Extended Experimental Procedures](#).

Preparation and Fractionation of Crude Reticulocyte Lysate

Reticulocytes were induced in rabbits and lysates were prepared and fractionated over DEAE cellulose to Fraction I (unabsorbed material) and Fraction II

genes in the xenografts. Dot sizes are as in (i). (iii) Selected annotation clusters most enriched for either up- or downregulated genes (above or below dashed line, respectively).

(B) (i) Enrichment analysis of consistently upregulated and downregulated transcripts for tumor suppressors and proto oncogenes annotations. (ii) Expression differences for all tumor suppressors (blue) and proto oncogenes (red) from (i). Gene names of the strongest differentially regulated cancer-related genes are shown.

(C) Integrated analysis of functional annotation clusters and known functional and physical protein-protein interactions among all consistently upregulated and downregulated genes (green and red, respectively). NF- κ B is shown in blue, and a close-up of the core interaction network surrounding NF- κ B (inset) is displayed. See also [Figure S5](#), [Tables S1](#) and [S2](#), and [Data S1](#).

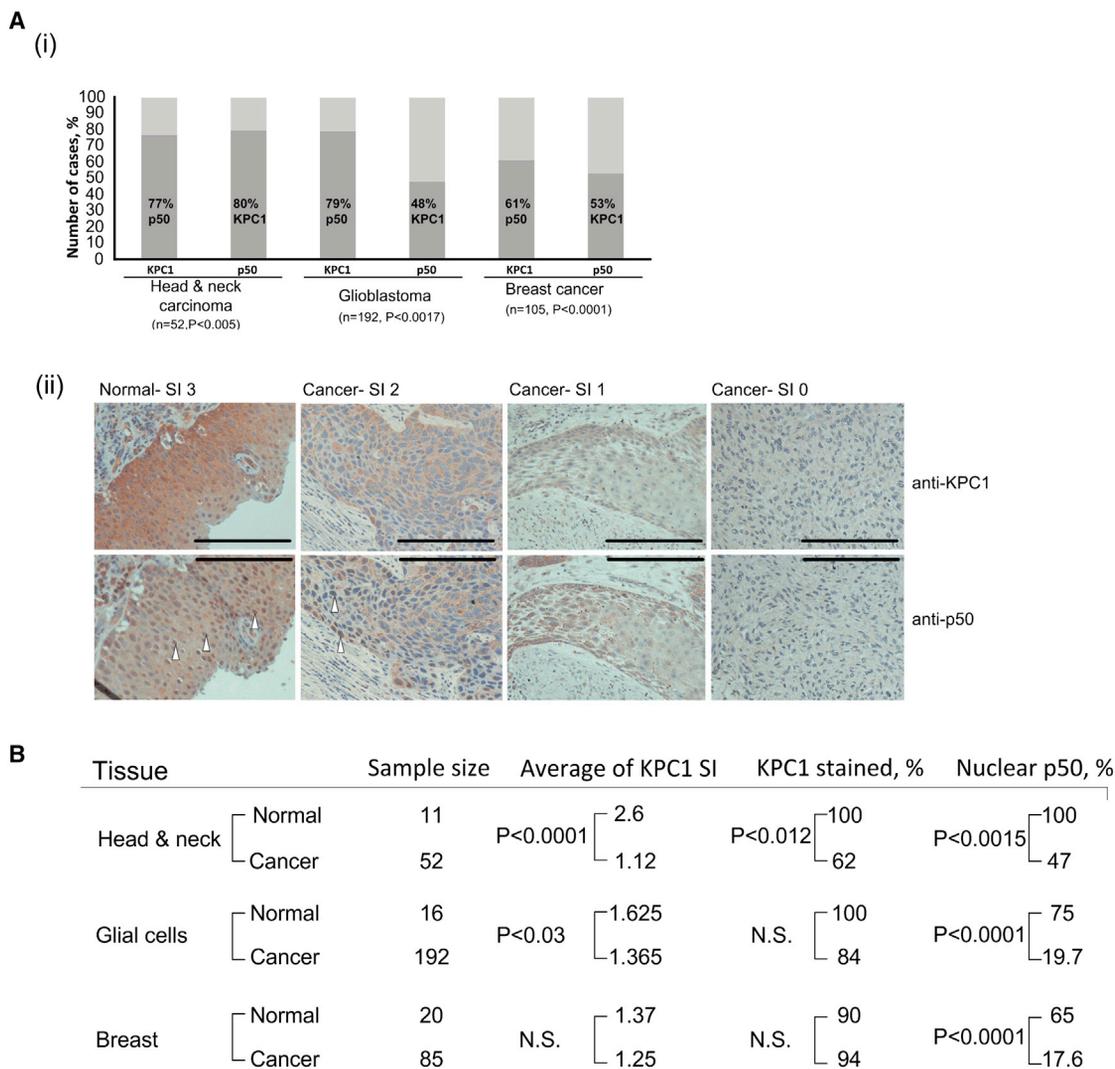


Figure 7. Correlation between the Expression of KPC1 and p50 in Tumoral and Normal Human Tissues

(A) (i) Correlation between expression of KPC1 and p50 in tumors. Immunohistochemistry of KPC1 and p50 in serial sections from SCCHN, and glioblastoma and breast cancer tissue arrays. P, p value. Analyses were carried out as described in the [Experimental Procedures](#). (ii) Representative immunostaining of SCCHN sections with anti-KPC1 or anti-p50. SI, staining intensity from 3 (strong positive) to 0 (negative). Arrowheads point to nuclear staining. Scale bars, 100 μ m.

(B) Statistical analysis of p50 and KPC1 staining in normal and cancerous head and neck, glial and breast tissues. “Average of KPC1 SI” represents mean of sample staining (number of samples is indicated under “Sample size”). “KPC1 stained, %” and “nuclear p50, %” represent percent of samples stained for KPC1 or nuclear p50. P, p value. The p value reflects the significance of difference between staining of normal and cancer tissue. SI, staining intensity; N.S., non-significant.

See also [Figure S6](#).

(high salt eluate) as described ([Hershko et al., 1983](#)). Fraction II (~200 mg) was further resolved using different successive chromatographic methods as described in the [Extended Experimental Procedures](#).

In Vitro Translation

p105 or p100 were translated in vitro in the presence of L-[³⁵S]methionine using the TNT T7 Quick reticulocyte lysate-based coupled transcription-translation kit according to the manufacturer’s instructions.

In Vitro Conjugation and Processing of p105

Ub conjugation and processing of ³⁵S-labeled p105 were carried out in a reconstituted cell-free system containing crude Fraction II as described

([Kravtsova-Ivantsiv et al., 2009](#)). For conjugation, 1 μ g of purified Kpc1-FLAG-TEV-6xHIS, Kpc1I1256A-FLAG-TEV-6xHIS, or 6xHis-KPC2 were added as indicated instead of Fraction II.

Ub Conjugates in Cells

HEK293 cells were transfected with control siRNA or siRNA against KPC1 as described above. After 24 hr, the cells were transfected with cDNAs coding for FLAG-p105 proteins along with cDNAs coding for HA-Ub and Myc-KPC1, or with an empty vector. After additional 24 hr, the proteasome inhibitor MG132 (20 μ M) was added for 3 hr, and the cells were lysed with RIPA buffer supplemented with freshly dissolved iodoacetamide and N-ethylmaleimide (5 mM each) to inhibit deubiquitinating enzymes. p105 (both free and

ubiquitinated) and free p50 were immunoprecipitated with immobilized anti-FLAG. The beads were washed five times with RIPA buffer and proteins were resolved by SDS-PAGE. Free and conjugated p105 (and free p50) were visualized using anti-FLAG.

Tumorigenicity

Cell-based (soft agar) and animal (mice xenografts) tumorigenicity assays are described in the [Extended Experimental Procedures](#).

RNA-Seq Analysis

RNA from U87-MG xenografts was isolated using RNA purification kit and analyzed using the Illumina HiSeq 2500 analyzer. Identification and clustering of the human genes are described in the [Extended Experimental Procedures](#).

Immunohistochemistry and Statistical Analysis

The staining technique and statistical analysis of the staining data of SCCHN, breast cancer, and glioblastoma were performed as described in the [Extended Experimental Procedures](#).

ACCESSION NUMBERS

The profile of gene expression in the tumors using RNA-seq analysis of transcripts reported in this paper is deposited in NCBI GEO under accession number GSE60530 and is available at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60530>.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, two tables, and one data file and can be found with this article at <http://dx.doi.org/10.1016/j.cell.2015.03.001>

AUTHOR CONTRIBUTIONS

A.C., T.S., and Y.T.K. contributed to the study design. Y.K.-I., I.S., and V.C.-K. contributed to the study design and carried out the biochemical and tumorigenicity assays. H.G. contributed to some of the biochemical experiments. T.Z. and A.A. performed the mass spectrometry analyses. I.N. and E.P. carried out the pathological observations. I.D. performed the statistical analyses of the stained human tumors. B.S. and G.S.-F. performed the computational analysis of the RNA-seq data. A.B. and M.J. synthesized the peptides and planned some of the experiments. A.C. supervised the study. Y.K.-I., I.S., and A.C. wrote the manuscript.

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